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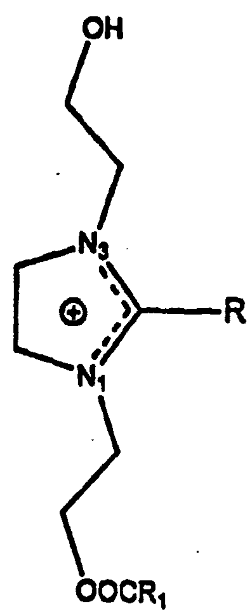
(54) Title: CATIONIC LIPID:DNA COMPLEXES FOR GENE TARGETING

(57) Abstract

The invention herein describes pharmaceutical compositions and methods for targeted delivery of functional genes into cells and tissues *in vivo*. The invention discloses DNA:lipid complexes, methods of making such complexes and methods of using such complexes for facilitating the targeted delivery and entry of recombinant expression constructs into cells and tissues *in vivo*, and particularly delivery of such recombinant expression constructs into cells and tissues *in vivo*, and particularly delivery of such recombinant expression constructs by intravenous, intraperitoneal or direct injection. The delivery vehicle for the targeting of the recombinant construct encoding the gene of interest, is composed of a mixture of a cationic imidazolinium lipid of formula (I) wherein R and R_1 mean a C_{11-29} aliphatic hydrocarbyl group, together with neutral lipids (e.g. DOPE, cholesterol).



(I)

CATIONIC LIPID:DNA COMPLEXES FOR GENE TARGETING

BACKGROUND OF THE INVENTION

1. Field of the Invention

A perennial goal in the pharmacological arts has been the development of methods and compositions to facilitate the specific delivery of therapeutic and other agents to the appropriate cells and tissues that would benefit from such treatment, and the avoidance of the general physiological effects of the inappropriate delivery of such agents to other cells or tissues of the body. Recently, the advent of recombinant DNA technology and genetic engineering has provided the pharmacological arts with a wide new spectrum of agents that are functional genes carried in recombinant expression constructs capable of mediating expression of these genes in host cells. These developments have carried the promise of "molecular medicine," specifically gene therapy, whereby a defective gene could be replaced by an exogenous copy of its cognate, functional gene, thereby alleviating a variety of genetic diseases.

However, the greatest drawback to the achievement of effective gene therapy has been the limited ability in the art to introduce recombinant expression constructs encoding functional genes into cells and tissues *in vivo*. While it has been recognized in the art as being desirable to increase the efficiency and specificity of administration of gene therapy agents to the cells of the relevant tissues, the goal of specific delivery has not been achieved in the prior art.

Liposomes have been used to attempt cell targeting. Rahman *et al.*, 1982, Life Sci. 31: 2061-71 found that liposomes which contained galactolipid as part of the lipid appeared to have a higher affinity for parenchymal cells than liposomes which lacked galactolipid. To date, however, efficient or specific delivery has not been predictably achieved using drug-encapsulated liposomes. There remains a need for the development of a cell- or tissue-targeting delivery system.

Thus there remains in the art a need for methods and reagents for achieving cell and tissue-specific targeting of gene therapy agents, particularly recombinant expression constructs encoding functional genes, *in vivo*.

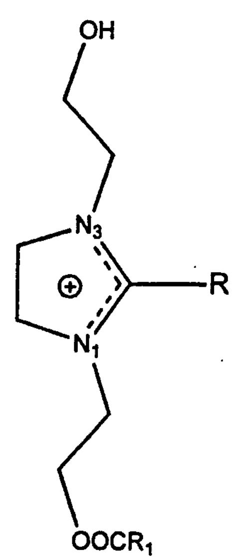
BRIEF SUMMARY OF THE INVENTION

The present invention is directed to improved methods for targeted delivery of functional genes to cells and tissues *in vivo*. This delivery system achieves such specific delivery by the formation of DNA:lipid complexes between nucleic acid

comprising a recombinant expression construct encoding a functional gene or fragment thereof complexed with a mixture of a cationic lipid and a neutral lipid. Methods of use are also provided. This invention has the specific advantage of targeted delivery of functional genes into cells *in vivo*, achieving effective intracellular delivery of constructs encoding functional genes more efficiently and with more specificity than conventional delivery systems.

In a first embodiment, the invention provides a pharmaceutical composition, comprising a formulation of a soluble complex of a recombinant expression construct and a mixture of a neutral lipid and a cationic lipid in a pharmaceutically acceptable carrier suitable for administration to an animal by injection. In this embodiment of the invention, the recombinant expression construct comprises a nucleic acid encoding a transcription product, the nucleic acid being operatively linked to gene expression regulatory elements and whereby the nucleic acid is capable of transcription *in vivo*. As used herein, the term "transcription product" is intended to encompass an RNA product resulting from transcription of a nucleic acid sequence, and explicitly includes RNA sequences that are not transcribed into protein (such as antisense RNAs or ribozymes), as well as RNAs that are subsequently translated into polypeptides or proteins.

In this first embodiment, the cationic lipid is a nitrogen-containing, imidazolinium-derived cationic lipid having the formula:



wherein each of R and R_1 independently is a straight-chain, aliphatic hydrocarbyl group of 11 to 29 carbon atoms inclusive. Preferred are those cations wherein each of R and R_1 independently have from 13 to 23 carbon atoms inclusive. In particularly preferred embodiments, the cationic lipid is 1-(2-(oleoyloxy)ethyl)-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride. In additional preferred embodiments, the neutral lipid is cholesterol, and the 1-(2-(oleoyloxy)ethyl)-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride and cholesterol are present in the complex at a molar ratio of 1:1. Further preferred embodiments comprise a recombinant expression construct encoding human CFTR and a mixture of a neutral lipid and a cationic lipid having a ratio of DNA to lipid of from about 1:6 to about 1:15 (μgDNA:nmoles lipid). Particularly preferred are embodiments where the DNA comprising the recombinant expression construct is present in the complex at a concentration of about 0.5 to 1mg/mL. In further preferred embodiments, the cationic lipid is 1-(2-(oleoyloxy)ethyl)-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride and the neutral lipid is dioleoylphosphatidyl ethanolamine, and the 1-(2-(oleoyloxy)ethyl)-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride and dioleoylphosphatidyl ethanolamine are present in the complex at a ratio of 1:1. Further preferred embodiments comprise a recombinant expression construct and a mixture of a neutral lipid and a cationic lipid having a ratio of DNA to lipid of about 1:1 (μgDNA:nmoles lipid). Particularly preferred are embodiments where the DNA comprising the recombinant expression construct is present in the formulation at a concentration of about 0.5 to 5mg/mL.

In a second embodiment, the invention provides methods for introducing a recombinant expression construct into a cell comprising lung tissue in an animal, the method comprising the step of administering the pharmaceutical composition of the invention to the animal by intravenous injection. In preferred embodiments, the cationic lipid is 1-(2-(oleoyloxy)ethyl)-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride. In additional preferred embodiments, the neutral lipid is cholesterol, and the 1-(2-(oleoyloxy)ethyl)-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride and cholesterol are present in the complex at a molar ratio of 1:1. Further preferred embodiments comprise a recombinant expression construct and a mixture of a neutral lipid and a cationic lipid having a ratio of DNA to lipid of from about 1:6 to about

1:15 (μgDNA:nmoles lipid). Particularly preferred are embodiments where the DNA comprising the recombinant expression construct is present in the formulation at a concentration of about 0.5-1mg/mL.

In another aspect of the second embodiment of the invention is provided methods for introducing a recombinant expression construct into a cell comprising spleen tissue in an animal, the method comprising the step of administering the pharmaceutical composition of the invention to the animal by intravenous injection. In preferred embodiments, the cationic lipid is 1-(2-(oleoyloxy)ethyl)-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride. In additional preferred embodiments, the neutral lipid is dioleoylphosphatidyl ethanolamine, and the cationic lipid and the neutral lipid are present in a molar ratio of 1:1. Further preferred embodiments comprise a recombinant expression construct and a mixture of a neutral lipid and a cationic lipid having a ratio of DNA to lipid of about 1:1 (μgDNA:nmoles lipid). Particularly preferred are embodiments where the DNA comprising the recombinant expression construct is present in the formulation at a concentration of about 1-2.5mg/mL.

In further embodiments of this aspect of the invention, the DNA:lipid complex is targeted to peritoneal macrophages by administration by intraperitoneal injection. In these embodiments, the cationic lipid is 1-(2-(oleoyloxy)ethyl)-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride, the neutral lipid is cholesterol, the cationic lipid and the neutral lipid are present in a molar ratio of about 1:1, the complex of a recombinant expression construct and a mixture of a neutral lipid and a cationic lipid has a ratio of DNA to lipid of about 1:1 (μgDNA:nmoles lipid), and the DNA concentration in the DNA:lipid formulation is about 1-2.5mg/mL. In additional embodiments of this aspect of the invention, the DNA:lipid complex is targeted to spleen macrophages and administered by intraperitoneal injection. In these embodiments, the cationic lipid is 1-(2-(oleoyloxy)ethyl)-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride, the neutral lipid is cholesterol, the cationic lipid and the neutral lipid are present in a molar ratio of about 1:1, the complex of a recombinant expression construct and a mixture of a neutral lipid and a cationic lipid has a ratio of DNA to lipid of about 1:1 (μgDNA:nmoles lipid), the DNA

concentration in the DNA:lipid formulation is about 1 to 2.5mg/mL.

In this aspect, the invention also provides methods for targeting gene transfer into pancreatic tissue by intraperitoneal injection. In preferred embodiments, the cationic lipid is 1-(2-(oleoyloxy)ethyl)-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride, the neutral lipid is dioleoylphosphatidyl ethanolamine, the cationic lipid and the neutral lipid are present in a molar ratio of about 1:1, the complex of a recombinant expression construct and a mixture of a neutral lipid and a cationic lipid has a ratio of DNA to lipid of about 1:1 (μgDNA:nmoles lipid), and the DNA concentration in the DNA:lipid formulation is about 1.5 to about 2.5mg/mL.

The invention also provides a method of introducing a recombinant expression construct into a cell comprising a tissue in an animal, the method comprising the step of administering the pharmaceutical composition of the invention to the animal by direct injection. In preferred embodiments, the cationic lipid is 1-(2-(oleoyloxy)ethyl)-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride and the neutral lipid is cholesterol. Also preferred are mixtures of the cationic lipid and the neutral lipid in a molar ratio of about 1:1. Preferred complexes include a complex of a recombinant expression construct and a mixture of a neutral lipid and a cationic lipid having a ratio of DNA to lipid of about 1:1 (μgDNA:nmoles lipid). The preferred DNA concentration in the DNA:lipid formulation is about 1-2.5mg/mL in this embodiment of the invention.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing the stability of DNA:lipid complexes of the invention assayed by intravenous administration and lung CAT assays over a period of 11 weeks.

Figure 2 is a graph of a comparison of chloride efflux in the presence and absence of stimuli in cells transfected with human CFTR-encoding plasmid vectors complexed with EDMPC:cholesterol.

Figure 3 is a schematic representation of the plasmid p4119.

Figure 4 is a histogram showing that mice administered CAT-encoding

of CAT-encoding DNA using DNA:lipid complexes of the invention.

Figure 17 is a histogram showing spleen-specific targeting by administration of CAT-encoding DNA using DNA:lipid complexes of the invention.

Figure 18 is a representation of tissue-specific targeting of CAT-encoding DNA complexed with different liposome complexes and administered intraperitoneally.

Figure 19 is a histogram showing CAT gene expression in human prostate tissue in which CAT-encoding DNA using DNA:lipid complexes of the invention were directly administered *ex corpora*.

Figure 20 is a histogram showing a comparison of spleen-specific and lung-specific targeting of DNA:lipid complexes of the invention using intravenous and intraperitoneal routes of administration.

Figure 21 shows the results of RT-PCR of transfected lung tissuse sections showing transgene sequences specifically targeted to vascular endothelial cells.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides compositions of matter and methods for facilitating the entry into cells of nucleic acids, particularly recombinant expression constructs encoding functional genes. For the purposes of this invention, the term "recombinant expression construct" is intended to encompass a replicable DNA construct comprising a nucleic acid encoding a functional gene or fragment thereof, operably linked to suitable control sequences capable of effecting the expression of the gene in a suitable host cell. Expressly intended to fall within the definition of a "gene" are embodiments comprising cDNA and genomic DNA sequences of functional genes, as well as chimeric hybrids thereof. Also intended to fall within the scope of the recombinant expression constructs of the invention are fragments or mutants of such genes which, when expressed, may inhibit or suppress the function of an endogenous gene in a cell, including, *inter alia*, *trans*-dominant mutants, antisense gene fragments and ribozymes.

In the recombinant expression constructs as provided by the present invention, the need for such control sequences will vary depending upon the host and cell types selected and the transformation method chosen. Generally, control sequences include

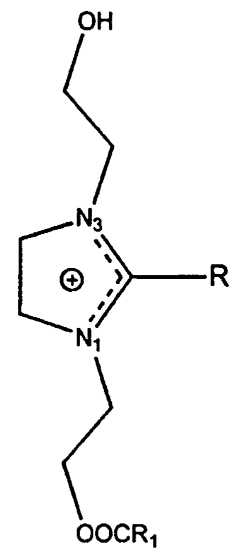
a transcriptional promoter, optional or ancillary transcription control sequences, such as transcription factor binding domains, enhancer sequences, and other eukaryotic "operator" sequences to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation. *See*, Sambrook *et al.*, 1990, Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Press: New York).

Vectors useful for practicing the present invention include plasmids, viruses (including phage), retroviruses, and integratible DNA fragments (i.e., fragments integratible into the host genome by homologous or non-homologous recombination). Also useful are vectors which replicate autonomously in host cells. Suitable vectors will contain replicon and control sequences which are derived from species compatible with the intended expression host cell.

The recombinant expression constructs of the present invention are useful in gene therapy, and specifically, for delivering exogenous, functional copies of a defective gene to a specific tissue target *in vivo*. *See generally* Thomas & Capecchi, 1987, Cell 51: 503-512; Bertling, 1987, Bioscience Reports 7: 107-112; Smithies *et al.*, 1985, Nature 317: 230-234.

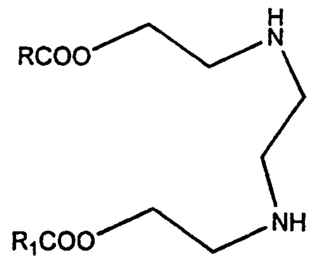
The invention provides complexes of recombinant DNA constructs encoding functional genes or fragments thereof and also comprising a mixture of a cationic lipid and a neutral lipid. For the purposes of this invention, the term "cationic lipid" is intended to encompass lipids which are positively charged at physiological pH, and more particularly, constitutively positively charged lipids comprising, for example, a quaternary ammonium salt moiety.

Specifically, the invention provides nitrogen-containing, imidazolinium-derived cationic lipids having the formula:

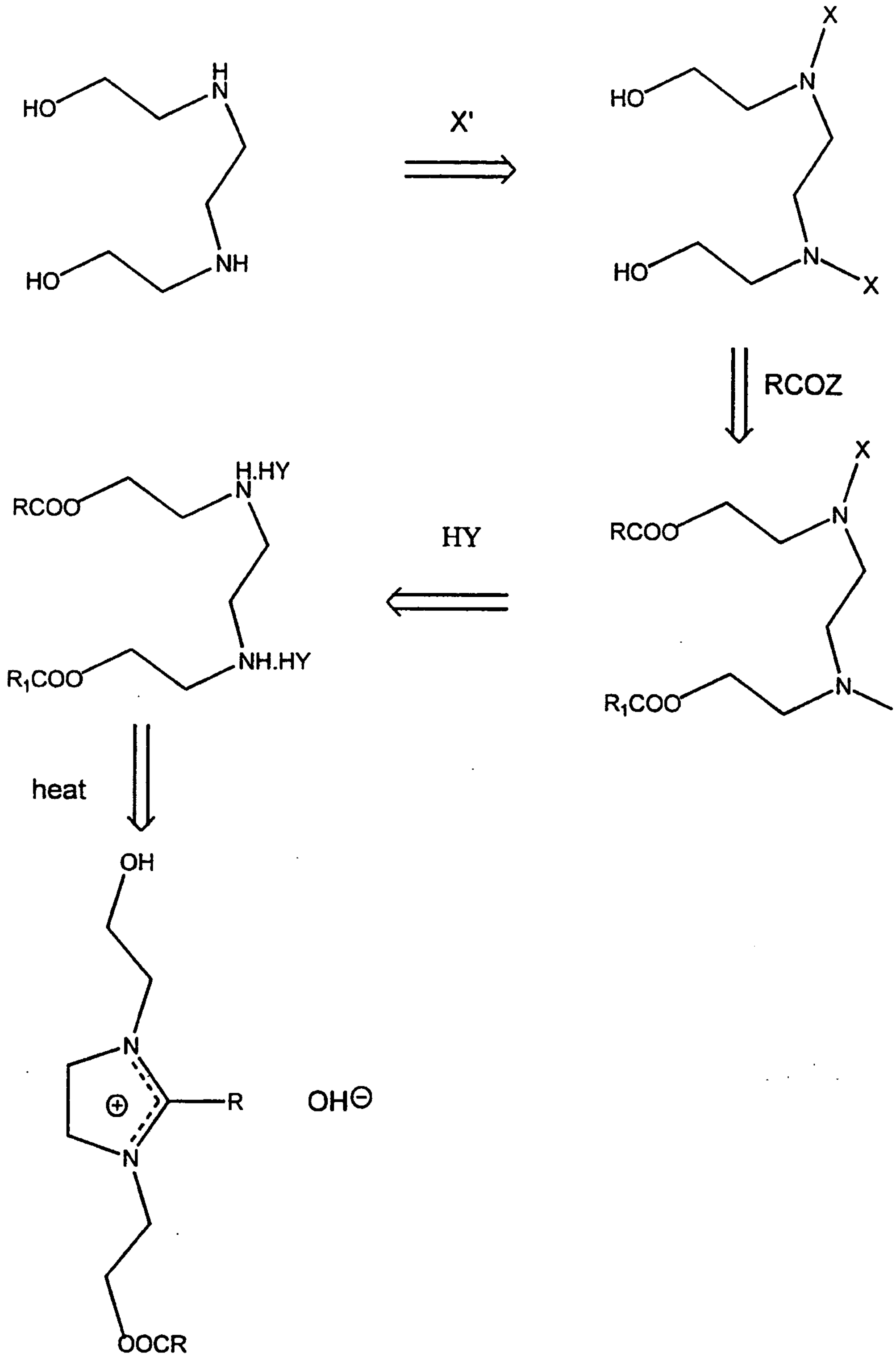


wherein each of R and R_1 independently is a straight-chain, aliphatic hydrocarbyl group of 11 to 29 carbon atoms inclusive. Preferred are those cations wherein each of R and R_1 independently have from 13 to 23 carbon atoms inclusive. The R and R_1 groups are saturated or are unsaturated having one or more ethylenically unsaturated linkages and are suitably the same or are different from each other. Illustrative R_1 groups include lauroyl, myristoyl, palmitoyl, stearoyl, linoleoyl, eicosanoyl, tricosanoyl and nonacosanoyl. In preferred embodiments, the cationic lipid is 1-(2-(oleoyloxy)ethyl)-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride (abbreviated as DOTIM herein).

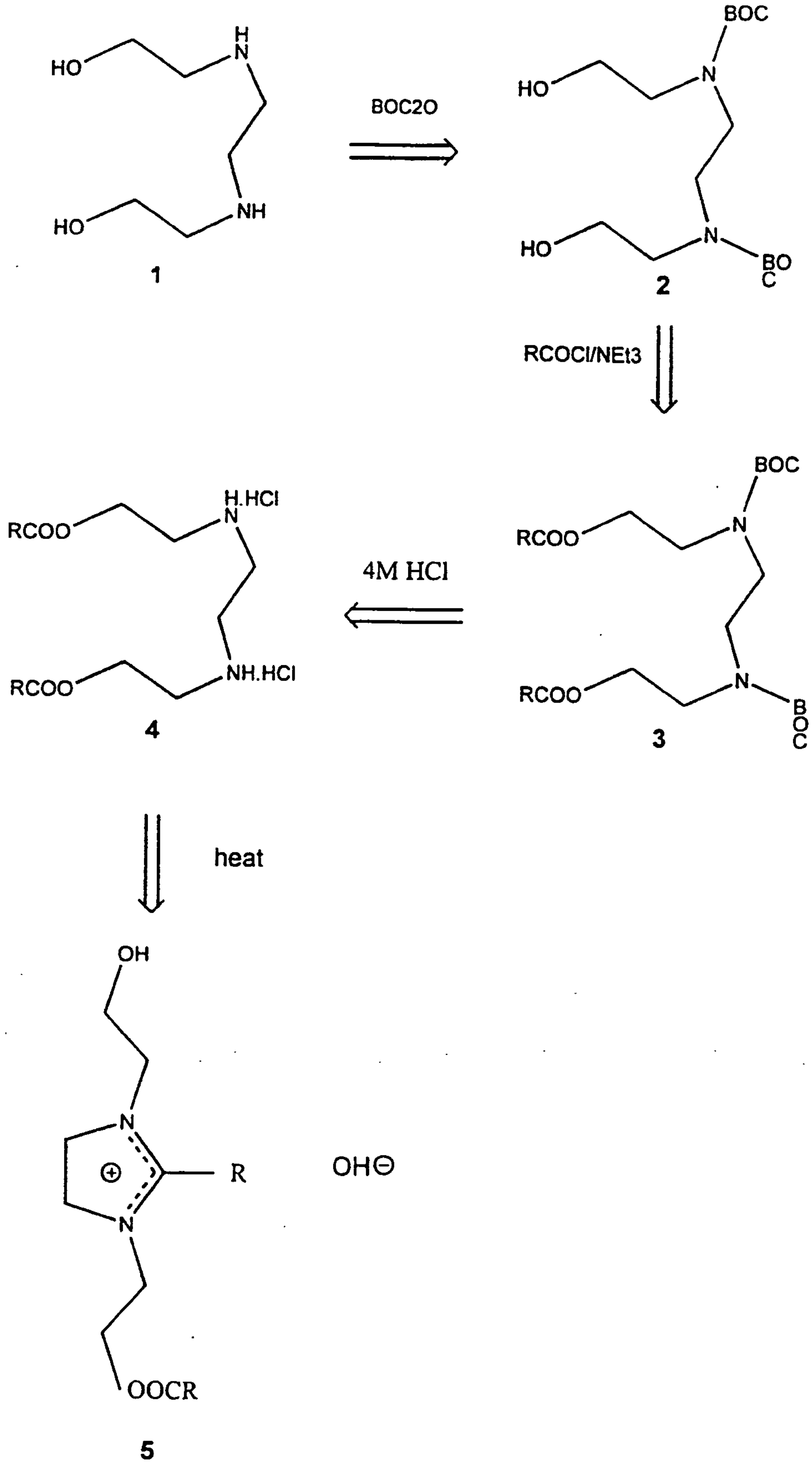
The cationic lipids comprising the liposome formulations of the invention can be synthesized by a rearrangement reaction. This reaction comprises synthesis of DOTIM from *N,N-bis*(2-hydroxyethyl)ethylene diamine through an amino-protected diacylated intermediate to the desired product. The method in general involves synthesis of an imidazolinium ion by heating a precursor compound of formula:



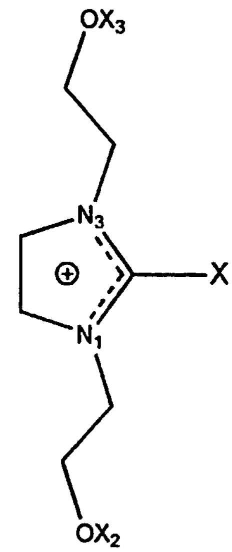
in an organic solvent at a temperature above the boiling point of water, wherein each of R and R_1 independently represents an organic group such that the precursor compound is soluble in the solvent and the R and R_1 are stable against reaction in the solvent at the temperature. Specifically, imidizolinium-comprising cationic lipids of the invention are prepared according to the following reaction scheme:


HO
HO
H
N
NH
X'
X
N
X
RCOZ
RCOO
R_1COO
H.HY
NH.HY
HY
heat
OH
R
$OH^{\ominus}$
OOCR

In this reaction scheme, X is any amino protecting group that reacts preferably with and protects a secondary amino group in the presence of a hydroxyl group, preferably one that is removable by acid hydrolysis (*e.g.*, with a strong acid such as HCL); X′ is the precursor of the X protecting group (*e.g.*, X′ is an anhydride or acid chloride where X is an acyl group); RCOZ is an acid chloride (Z is Cl) or anhydride (Z is RCOO) in which R is defined as either R or R_1; and HY is a strong acid (*e.g.*, sulfuric acid or a derivative thereof or a hydrogen halide). A preferred amino protecting group is *t*-butyloxycarbonyl (from di-*t*-butylpyrocarbonate). Preferred acylating groups are acid chlorides of fatty acids (such as the fatty acid substituents of the imidazolinium herein described). A preferred acid for the rearrangement and deprotection steps of the preparative scheme disclosed above (and which can be combined in a single step) is HCL. Heat for the rearrangement reaction is preferably provided by reflux in a solvent having a boiling point in the range of 100° to 200°C, more preferably in the range of 100°-150°C. The initial imidizolinium ion is formed as a hydroxide salt and/or a chloride salt (if HCL is used as the strong acid), but the anion can be replaced by an exchange reaction. This specific reaction scheme is shown below:


HO
H
N
HO
NH
1
BOC2O
BOC
HO
N
HO
N
BOC
2
RCOCl/NEt3
BOC
RCOO
N
RCOO
N
BOC
3
4M HCl
H.HCl
RCOO
N
RCOO
NH.HCl
4
heat
OH
N
⊕
R
N
OH⊖
OOCR
5

This synthetic scheme is not limited to the explicitly-disclosed imidazolinium compounds comprising the formulations of the invention. This reaction scheme provides a general protocol for the production of imidazolinium compounds of formula:



in which X_1 represents the residue of an acyl group after the rearrangement reaction as shown (from H to complex organic group) while X_2 and X_3 independently represent H or an organic group. X_2 would initially represent R-CO-, but this group could be removed or be replaced by a different organic group using standard chemical reactions; since one of the two potential hydroxyl groups in the initial product is already protected, synthesis of compounds in which X_2 and X_3, represent different groups can readily be accomplished. Ions in which both X_2 and X_3 represent H are preferred, as these can be used in the synthesis of numerous imidazolinium compounds. Although there is no particular limit on the structure of the three "X" groups in the general synthesis other than those imposed by solubility or reactivity under the heating conditions being used for the reaction (which will be readily apparent), preferred organic groups are hydrocarbyl groups containing 30 or fewer carbons and their oxygenated products (especially fatty acids and their reaction products as previously described, as well as other hydrocarbyl groups and oxygenated products containing 15 or fewer carbon atoms, preferably 10 or fewer, more

preferably hydrocarbyl groups containing no more than one phenyl ring with the remainder of the hydrocarbyl group being composed of alkyl groups, especially alkyl groups of 5 or fewer carbons). Organic groups formed oxygenated hydrocarbyl groups are preferably carboxylic acids, alcohols, esters, ethers, ketones and aldehydes containing no more than one such functional group per organic group. Examples of imidazolinium ions that can be prepared by the synthesis as described above (with further modification of the hydroxyl groups using simple organic reactions) include 1,3-dihydroxyethylimidazolinium, 1-methoxyethyl-3-hydroxyethylimidazolinium, 1-hydroxyethyl-2-phenyl-3-methylcarboxyethylimidazolinium, 1,3-dimethoxyethoxy-ethylimidazolinium, 1,3-hydroxyethyl-2-tridecylimidazolinium, and 1-hydroxyethyl-2-cis, cis-8,11-heptadecyldienyl-3-oleoyloxyethylimidazolinium.

Since the reaction is a simple self condensation reaction with the elimination of water, the solvent and/or other reaction conditions are not important to the overall reaction. Any solvent can be used that will dissolve the precursor compound and that has a boiling point above that of water (under the pressure conditions of the reaction, which are not limiting). If an acid catalyst is used to speed up the reaction, a protic solvent is preferred in order to provide easier proton exchange. Ethylene glycol and other alcohols having a boiling point above 100°C are preferred.

One of the explicitly-disclosed cationic lipids of the invention (termed DOTIM) is also commercially available (Avanti Polar Lipids, Alabama).

Cationic lipids are particularly useful as carriers for anionic compounds, particularly polyanionic macromolecules such as nucleic acids. As cationic lipids are positively charged, a tight charge complex can be formed between a cationic lipid carrier and a polyanionic nucleic acid, resulting in a lipid carrier-nucleic acid complex which can be used directly for systemic delivery to a mammal or mammalian cell.

Neutral lipids, in contrast to the cationic lipids of the invention, are characterized as being electrochemically neutral, although this definition does not preclude protonation of such lipids to produce a positively-charged salt under certain conditions. Expressly included within this definition are, *inter alia*, steroids such as cholesterol and dioleoylphosphatidyl ethanolamine (DOPE).

Complexes of DNA and mixtures of cationic and neutral lipids of the invention

are characterized by a number of parameters intrinsic to the formation of such complexes. These include the identity of the cationic lipid and the neutral lipid; the ratio of cationic lipid to neutral lipid; concentration of DNA in the complex; the ratio of DNA to lipid; DNA purity; cationic liposome size; methods of preparing liposomes; the methods of preparing the DNA:lipid complexes; and other variables. Preferred combinations of cationic and neutral lipids include 1-(2-(oleoyloxy)ethyl)-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride and cholesterol and 1-(2-(oleoyloxy)ethyl)-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride and dioleylphosphatidyl ethanolamine. A preferred molar ratio of these lipids is 1:1. DNA concentration in the formulations of the invention is from about 0.5mg/mL to about 5mg/mL, more preferably from about 0.5mg/mL to about 2.5mg/mL. DNA:lipid ratios are preferably from about 1:1 (μg DNA/nmole lipid) for formulations to be injected intraperitoneally or by direct injection, to from about 1:6 to 1:15 (μg DNA/nmole lipid) for preparations to be injected intravenously. DNA purity has a direct effect on liposome complex formation, but DNAs having a purity of about 15% to about 100% are appropriate for complex formation. DNAs having a purity of 90-100% by HPLC are preferably used in DNA:lipid complexes in a range of 1:12 to 1:15 μg DNA/nmole lipid.

The various lipid carrier-nucleic acid complexes, wherein the lipid carrier is a liposome, are prepared using methods well known in the art. Mixing conditions can be optimized by visual examination of the resultant lipid-DNA mixture to establish that no precipitation or aggregation occurs. To make the lipid-DNA complexes more visible, the complexes can be stained with a dye which does not itself cause aggregation, but which will stain either the DNA or the lipid. For example, Sudan black (which stains lipid) can be used as an aid to examine the lipid-DNA mixture to determine if aggregation has occurred. Particle size also can be studied with methods known in the art, including electronic microscopy, laser light scattering, Coulter™ counting/sizing, and the like. Standard-size beads can be used to calibrate instruments used for determining the size of any liposomes or complexes that form.

By "lipid carrier-nucleic acid complex" is meant a nucleic acid sequence as described above, generally bound to a lipid carrier preparation, as discussed below. The lipid carrier preparation can also include other substances or cofactors.

Furthermore, the lipid carrier-nucleic acid complex can include targeting agents to deliver the complex to particular cell or tissue types. Generally, the nucleic acid material is added to a suspension of preformed liposomes which may be multi-lamellar vesicles (MLVs) or small unilamellar vesicles (SUVs), usually SUVs formed by sonication or by extravasation through appropriately-sized polycarbonate membranes. The liposomes themselves are prepared from a dried lipid film that is resuspended in an appropriate mixing solution such as sterile water or an isotonic buffer solution such as 10mM Tris/NaCl or 5% dextrose in sterile water and sonicated to form the liposomes. Then the preformed lipid carriers are generally mixed directly with the DNA.

Mixing and preparing of the lipid-DNA complex can be critically affected by the sequence in which the lipid and DNA are combined. Generally, it is preferable (to minimize aggregation) to add the lipid to the DNA at ratios of DNA:lipid of up to 1:2 inclusive (microgram DNA:nanomoles cationic lipid). Where the ratio of DNA:lipid is 1:4 or higher, better results are generally obtained by adding the DNA to the lipid. In either case, mixing should be rapidly achieved by shaking or vortexing for small volumes and by use of rapid mixing systems for large volumes. The lipid carrier and DNA form a very stable complex due to binding of the negatively charged DNA to the cationic lipid carriers. The DNA:lipid complexes of the invention find use with small nucleic acid fragments as well as with large regions of DNA (≥30kb).

Aggregation of the lipid carrier-nucleic acid complex is prevented by controlling the ratio of DNA to lipid carrier, minimizing the overall concentration of DNA:lipid carrier complex in solution (usually less than 5 mg DNA/mL solution) and avoiding the use of chelating agents such as EDTA and/or significant amounts of salt, either of which tends to promote macro-aggregation. The preferred excipient is water, dextrose/water or another solution having low or zero ionic strength. Further, the volume should be adjusted to the minimum necessary for injection into the host mammal, while at the same time taking care not to make the solution too concentrated so that aggregates form.

DNA:lipid complexes of the invention may be sized in accordance with conventional techniques, depending upon the desired size. For intravenous or

intraperitoneal delivery, the complexes of the invention are preferably 150-300nm in diameter.

The DNA:lipid complexes of the invention have utility in mediating the efficient delivery of the recombinant expression constructs of the invention, encoding functional genes of fragments thereof, into eukaryotic, preferably mammalian, most preferably human cells. DNA:lipid complexes of the invention are useful for achieving gene transfer *in vitro* using established techniques. More importantly, the DNA:lipid complexes provided by this invention, and the methods of administering the DNA:lipid complexes provided herein, are capable of specifically delivering recombinant expression constructs of the invention to particular tissues and cells comprising those tissues *in vivo*, thereby providing targeting of these genes to specific tissues. These properties of the pharmaceutical compositions and methods of the present invention provide for realization of practical gene therapy, *whereby, e.g.*, a particular deficient gene is restored by the introduction of a functional copy of the normal cognate gene into the cells of the affected tissue, without the inappropriate introduction of the construct into other cells and tissues of the body nonspecifically.

Thus, the invention provides methods and pharmaceutical compositions having a number of advantages over the prior art. The liposomes and lipid complexes of the invention have been extensively studied in humans, and are non-immunogenic, relatively non-toxic, and non-infectious. These complexes are stable, as illustrated by the experimental results shown in Figure 1. A particular DNA:lipid complex (DOTIM:Cholesterol (1:1) complexed with a CAT-encoding plasmid at a DNA:lipid ratio of 1:6 and a DNA concentration of 0.625mg/mL) was prepared and tested weekly over 11 weeks by injection into the tail vein of ICR mice. CAT activity was then determined in mouse lung using protocols described in detail below. The Figure shows results demonstrating that this preparation was stable over the course of the experiment, whereby substantially identical levels of CAT gene expression were obtained at all time points tested.

The DNA:lipid complexes of the invention have additional advantages over the prior art. Recombinant expression constructs of any practicable size can be used, there being no limitation on large plasmid size due to the absence of packaging the

DNA into the genome of a vector organisms like a retrovirus or an adenovirus. Gene transfer can be achieved in non-dividing cells, unlike prior art systems which relied on viral vectors whose life cycle required the infected cells to be dividing. In addition, the specific formulation of the DNA:lipid complexes of the invention can be altered to affect targeting and duration of the gene-expression effect. The DNA:lipid complexes of the invention are also amenable to many delivery routes, and are less likely to encounter the types of safety issues related to viral-based delivery systems.

The DNA:lipid complexes of the invention may be administered to an animal to effect delivery of functional genes into specific tissues by any appropriate therapeutic routine, including intravenous, intraperitoneal, subcutaneous, or intramuscular injection, as well as direct injection into target tissue(s). Typically, the DNA:lipid complexes of the invention are injected in solution where the concentration of the DNA to be delivered dictates the amount of the complex to be administered. This amount will vary with the tissue to be targeted and the effectiveness of the targeted DNA, the required concentration for the desired effect, the number of administrations, and the like.

The methods and pharmaceutical compositions of the invention are particularly useful and appropriate for introducing functional human genes, particularly human CFTR, to lung tissue. These methods and pharmaceutical compositions thus have utility in the treatment of human diseases, including cystic fibrosis and chronic bronchitis.

The following Examples illustrate certain aspects of the above-described methods and advantageous results. The following examples are shown by way of illustration and not by way of limitation.

EXAMPLE 1

Preparation of DOTIM:Cholesterol (1:1) Small Unilamellar Vesicles

To a 1L round bottom flask was added 500 μmoles cholesterol dissolved in an excess of chloroform and then 500 μmoles DOTIM also dissolved in an excess of chloroform. The amount of DOTIM was determined by high pressure liquid chromatography (HPLC) or be UV spectroscopy at 237nm.

After brief, gently mixing, the flask was attached to a rotary evaporating apparatus and chloroform withdrawn under slow speed and water vacuum conditions until almost all of the solvent was evaporated. Evaporation was completed at maximum rotation speed using a vacuum pump to completely dry the lipid mixture to a thin film on the wall of the round bottom flask.

As an intermediate step to the formation of the title composition, multilamellar vesicles (MLVs) were prepared from this film by the addition of 16mL endotoxin-free water to the flask, which was then warmed to 37°C in a water bath with gentle hand-swirling. The MLVs thus formed were removed from the flask using a 9" Pasteur pipette and transferred to a 20mm screw cap tube at room temperature. The flask was cleared of any remaining MLVs by washing with an additional ml endotoxin-free water, which was added to the 16mL previously transferred from the flask. These solutions were mixed, and aliquotted equally into 20 16mL screw cap tubes using a Pasteur pipette.

MLVs were converted into the SUVs of the title composition by sonication. Each of the 16mL screw cap tubes containing MLVs were placed individually into a sonicating water bath maintained at 36°C for 5 min, and the temperature of the bath checked between the introduction of each tube. Sonicated droplets within each tube were collected by brief vortex mixing, and the individual solutions of SUVs were then combined into a single 20mm screw cap tube using a 9" Pasteur pipette, and then filtered using a 0.2 micron disposable filter (Nalgene). Finally, an amount of an endotoxin-free solution of 25% dextrose in water, equal to one-quarter of the final volume of SUVs, was added to the tube of SUVs. This resulted in a suspension of SUVs comprising 20mM DOTIM and 20mM cholesterol (40mM total lipid) in a 5% dextrose solution, which was kept at 4°C until use.

EXAMPLE 2

Large Scale Plasmid DNA Preparation

Plasmid DNA was prepared in large-scale (*i.e.*, milligram) quantities using a modification of the alkaline lysis procedure (Sambrook *et al.*, 1990, *ibid.*). Briefly, bacteria comprising a single colony were grown for 12-18 hours or overnight in 15mL

TB broth (47g/L TB (Sigma Chemical Co., St. Louis, MO)/ 8% glycerol) supplemented with 100μg/mL carbenicillin at 37°C with shaking (250 rpm). 2-2.5mL of this culture was then added to 400mL TB (supplemented with 100μg/mL carbenicillin) in each of six 2L flasks (for a total of 2.4L culture) and grown at 37°C with shaking overnight (16-18h).

After overnight growth, bacteria were collected by centrifugation for 10 min. at 4°C in a Beckman J2-MI centrifuge equipped with a JA-10 rotor. The bacterial pellet in each centrifuge bottle was gently resuspended in 20mL of an ice-cold solution of 50mM dextrose in 25mM HCL buffer (pH8)/10mM EDTA. To the resuspended bacterial cell pellets were added 40mL of a freshly-made solution of 0.2N NaOH/1% sodium dodecyl sulfate at room temperature, resulting in cell lysis upon gentle agitation of this mixture on ice for about 5 min. After the added lysis solution has been thoroughly mixed into the bacterial suspension and the cells lysed, the mixture was allowed to stand at room temperature for 5 min. To this mixture of lysed bacteria was added 20mL of an ice-cold solution of 3M potassium acetate, which was mixed into the lysed bacterial solution gently by hand and then stored on ice for 10 min. A flocculant white precipitate formed, comprising bacterial chromosomal DNA, RNA and SDS/protein/membrane complexes, which were cleared from the solution by centrifugation at 8000rpm for 15 min at 4°C in the JA-10 rotor as above.

After centrifugation, the supernatant was transferred with filtering through Miracloth to 250mL centrifuge bottles, and 50mL isopropanol added at room temperature, mixed and incubated for 10 min. The plasmid DNA precipitate was recovered by centrifugation at 5000rpm for 10min at room temperature in a JA-14 rotor (Beckman). The alcohol-containing supernatant was decanted and residual supernatant removed by vacuum aspiration.

The plasmid DNA pellets were resuspended in 6mL of a solution of 6mM Tris-HCL (pH8) and transferred to 50mL centrifuge tubes upon dissolution. To each tube was added and equal volume of cold (-20°C) 5M LiCl, the solutions mixed by hand and then centrifuged at 8000rpm for 10min at room temperature in a JA-20 rotor (Beckman). The supernatant solution from each tube was transferred to a fresh tube and the plasmid DNA then re-precipitated by the addition of an equal volume of

isopropanol, mixed and collected by centrifugation at 5000rpm for 10min at room temperature in a JA-20 rotor. The alcohol-containing supernatant solution was then decanted, residual alcohol removed by aspiration, and the plasmid DNA pellets allowed to air dry for 5min.

Contaminating bacterial RNA was removed from the plasmid DNA by dissolving the pellets in 1mL 10mM Tris-HCL (pH8), adding about 0.5-0.75μg of pancreatic RNase per mL, followed by incubating the mixture at 37°C for 1h. Disappearance of RNA was determined by ethidium bromide-stained agarose gel analysis (*see* Sambrook *et al.*, *ibid.*). Plasmid DNA was purified by phenol-chloroform extraction. Briefly, to each aliquot of plasmid DNA solution was added an equal volume of Tris-saturated phenol:chloroform (1:1), the immiscible solutions mixed by vortexing, and centrifuged in a laboratory tabletop microfuge for 5min at room temperature. The aqueous (upper) layer was removed, transferred to a fresh microfuge tube, and extraction with phenol:chloroform repeated at least twice. These extractions were followed by two extractions of the aqueous layer with Tris-saturated chloroform. Plasmid DNA was concentrated by precipitation, with the addition of 5M sodium acetate to a final concentration of 0.3M and the addition of two volumes of cold (-20°C) absolute ethanol. DNA was allowed to precipitate in this solution at -20°C for 1h or overnight.

After precipitation, plasmid DNA was collected by centrifugation at about 6000rpm in a clinical microcentrifuge. The alcohol-containing supernatant was aspirated by vacuum, and the pellet washed twice with 70% ethanol/water (4°C). The washed pellets were air dried for at least 30min. Plasmid DNA pellets were dissolved in a total of 6mL of a solution of 10mM Tris-HCL (pH8), and concentration determined by spectrophotometric analysis of a 1-to-200 dilution of the recovered plasmid at A_{260}.

EXAMPLE 3

Preparation of DNA:LIPID Complexes

DOTIM:cholesterol:plasmid DNA liposomes were prepared as follows. A DOTIM:cholesterol mixture (1:1, 20μmoles/μL each lipid) was prepared as described

in Example 1 above. Complexes with plasmid DNA were prepared in DNA:lipid ratios of 1:1 and 1:6. DNA and DOTIM:cholesterol were each first brought from storage conditions (-20°C for DNA, 4°C for liposome formulations) to room temperature before use over the course of about 1.5h. DNA concentration in the complex preparations were optimally 100-550μg/200μL complex solution (for ratios of 1:1 DNA:lipid) and 100-150μg/μL complex (for ratios of 1:6 DNA:lipid). DNA concentrations were typically determined just prior to DNA:lipid complex formation, by ultraviolet spectrophotometry as described in Example 2. DOTIM:cholesterol mixtures were typically used at a total lipid concentration of 40μmole/mL, corresponding to 20μmole/mL DOTIM and 20μmole/mL cholesterol.

DNA:lipid complexes were prepared from these reagents as follows. Each component was prepared in individual microfuge tubes to a total volume per tube of 100μL. An appropriate amount of DNA (equivalent to a final DNA concentration of 500μg DNA/mL complex) was added to one tube, and brought to volume with water or a solution of 5% dextrose in water. The appropriate amount of the DOTIM:Cholesterol mixture (100nmoles lipid/100μg DNA at a 1:1 ratio; 600nmoles lipid/100μg DNA at a 1:6 ratio) was added to a second tube, and water or a solution of 5% dextrose in water was added to bring this solution to a total volume of 100μL. The contents of the lipid-containing tube were mixed by vortexing for about 2sec, while the contents of the DNA-containing tube were mixed gently using a 1mL pipettor. The contents of the lipid mixture-containing tube were then added to the DNA-containing tube using a 1mL pipettor. It was found that it was essential that this addition was performed slowly, in a constant stream, to the top of the DNA solution in tube A. As the lipid solution mixed with the DNA, formation of the DNA:lipid complex was detected by the solution becoming slightly cloudy and opalescent. It was also determined that, at this stage, the mixture could not be vigorously mixed (for example, by vortexing) without seriously compromising the integrity and usefulness of the complexes so formed; however, it was advantageous to gently mix the entire contents of the tube 3-4 times after completion of addition of the lipid mixture to the DNA mixture.

After the complexes were formed, the final concentration of DNA was

determined by ultraviolet spectrophotometry as described above, and the size of the DNA:lipid complexes determined by light scattering measured at 400nm.

EXAMPLE 4

Preparation of Tissue Samples for CAT Assay and Protein Determination

Tissues were prepared for assay as follows. Experimental animals were euthanized quickly and humanely. Mice were typically placed in a kill box flooded with CO_2 for 2-3 min. Tissues were harvested by dissection and weighed, and then placed in 1mL cold homogenation buffer (250mM Tris/5mM EDTA) supplemented with PMSF (35μg/mL) and Leupeptin/Aprotinin (5μg/mL). Tissues were then homogenized for 20-30 sec using a tissue disruptor (such as a Polytron) until a uniform homogenate was obtained. This homogenate was then transferred to a centrifuge tube and quickly frozen on dry ice (or at -70°C) and then thawed at 37°C. Insoluble debris was cleared from the homogenate by centrifugation at 10,000*g* for 5-10 min. 50μL of the resulting supernatant solution was aliquotted into a microfuge tube and stored at -70°C until used for protein determinations.

The remainder of the supernatant was heat inactivated at 65°C for 15 min and re-centrifuged at 10,000*g* for 10min, and stored at -70°C until use for CAT assay determinations.

To perform CAT assays, samples were analyzed in parallel with a series of standard CAT activity samples. From the standards was developed a standard curve of CAT activity *versus* CAT protein, which was used to determine the level of CAT protein expression in tissue samples based on the observed CAT activity in tissue homogenates. To prepare the standard curve, serial dilutions of CAT enzyme were prepared ranging from 0.1 to 0.000025U. These standards were prepared in a reaction mixture consisting of 50μL BSA buffer (having formula: 250mM Tris/5mM EDTA/2mg/mL BSA, Fraction V (U.S. Biochemical, Cleveland, OH), 5μL standard CAT enzyme (and appropriate dilutions; obtained from Sigma Chemical Co, St. Louis, MO), 50μL ^{14}C-labeled chloramphenicol (New England Nuclear; diluted 1:10 in BSA buffer prior to use) and 25 μL n-butyryl-CoA (Sigma). Tissue samples were prepared identically, with the exception that 30μL of tissue homogenate was substituted for the

5μL of standard CAT enzyme activity. Samples were incubated at 37°C for 2h. After this incubation, 300μL of mixed xylenes (Aldrich Chemical Co.) were added to each tube, vortexed for 30sec, and centrifuged for 3min at 10,000rpm in an IEC centrifuge equipped with a 24-slot rotor. The mixed xylenes (upper) phase of each sample tube was transferred to a fresh microfuge tube and 750μL homogenation buffer added. The samples were then vortexed and centrifuged as described above.

200μL of the upper phase from each tube were transferred to liquid scintillation vials and 0.5mL scintillation cocktail (Ready-Safe, Beckman) added. The amount of CAT-specific radioactivity in each sample was determined by liquid scintillation counting assay.

EXAMPLE 5

X-Gal Staining of Tissue Samples

Tissue samples were stained with X-gal (5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside) using the following protocol. Tissues are fixed by immersion for 0.5-1h on ice in freshly-made fixative solution (2% neutral buffered formalin/0.02% gluteraldehyde/0.02% Nonidet-P40). After fixation, tissues were rinsed twice at 4°C in a solution of 2mM $MgCl_2$/0.1% desoxycholate/0.2% NP-40 in 10mM phosphate-buffered saline (PBS; pH 7.3). Tissues were then stained using rinse solution supplemented with 1mg/mL X-Gal (U.S. Biochemical), 5mM ferricyanide and 5mM ferrocyanide. Tissues were stained for 12-48h at 37°C or room temperature. After staining, tissues are rinsed in PBS. Tissues were then frozen and sectioned or fixed in 70% ethanol, embedded in paraffin and sectioned.

Protein determinations were performed using a dye binding assay (BSA Protein Assay Reagent, Pierce Chemical Co.). The Pierce reagent was prepared by mixing 50 parts of Reagent A with 1 part Reagent B as provided by the supplier. 100μL of this prepared reagent were aliquotted into each well of a 96 well microtitre plate. 100 μL of a solution containing 20 μg BSA were added to the first well of the first row (*i.e.*, well A1) and 100μL of a 1:2 to 1:8 dilution of each tissue extract were added to the other wells in the row. Serial dilutions at ratios of 1:2 were made in each of the adjoining rows consecutively using the wells in the preceding row.

Typically, 96-well plates having 12 wells/row resulted in 6 serial dilutions (1 to 1/64); the last row was a blank loaded with PBS as a control. The plates were incubated at 37°C for 30 minutes, and the extent of dye binding determined spectrophotometrically as absorbance at 562nm. Protein concentrations in sample wells were determined in comparison with a standard curve generated using the OD readings from the serial dilutions of the BSA standard.

EXAMPLE 6

Microplate Assay for β-galactosidase Expression in Tissues

Tissue was homogenized in an appropriate volume of homogenization buffer (250mM Tris/5mM EDTA) (*e.g.*, 300μL were used to homogenize a mouse lung). The homogenate was then incubated on ice for 30min and centrifuged in a microcentrifuge for 10min at 13,000rpm to clear the homogenate of insoluble debris. Supernatants from these homogenates were collected and assayed as follows.

Microplates were prepared for these analyses as follows. For each plate to be covered, 50μL of anti-β-galactosidase monoclonal antibody was diluted in 5mL of 50mM sodium bicarbonate buffer (pH 9.4). 50μL of the diluted antibody solution was added to each well of a microtitre plate (*e.g.*, Immulon 3, Dynatech), the plate sealed and incubated overnight at 4°C. After overnight incubation, 200μL BLOTTO solution (5% v/v nonfat dry milk and 0.2% Tween-20 in PBS) were added to each well and incubated for 1h at room temperature. The BLOTTO solution was then removed and the plates washed three times with a solution of PBS/0.2% Tween-20, with the exception that the first row was not washed with this solution. 100μL of a standard solution of 10mU/mL β-galactosidase were added to the first well of the first row (*i.e.*, well A1) and 100μL of each tissue extract were added to the other wells in the row. Serial dilutions at ratios of 1:2 were made in each of the adjoining rows consecutively using the wells in the preceding row. Typically, 96-well plates having 12 wells/row resulted in 6 serial dilutions (1 to 1/64); the last row is a blank loaded with PBS as a control. The plates were incubated at room temperature for 1h, and then washed three times with a solution of PBS/0.2% Tween-20 as above.

To develop the assay, 100μL of CPRG assay buffer (2.5mg/mL chlorphenol

red-β-D-galactopyranoside monosodium salt (CPRG)/1.8mg/mL $MgCl_2$/7.1μL/mL 2-mercaptoethanol in PBS) were loaded into each washed well and the plates then incubated at 37°C for 2h. The extent of β-galactosidase expression was then determined spectrophotometrically as absorbance at 562nm.

Whole tissues and tissue sections were assayed using a modification of this protocol. Frozen tissue or tissue sections were fixed by immersing the frozen tissues in fixative solution (2% neutral buffered formalin/0.02% glutaraldehyde/0.02% NP-40) without thawing. Tissues were incubated in fixative solution for 2h at room temperature with gentle agitation. After incubation, the tissues were rinsed twice with PBS, then incubated at 37°C overnight in X-Gal staining solution (5mM potassium ferricyanide/5mM potassium ferrocyanide/0.01% sodium desoxycholate/0.02% NP-40/1mg/mL X-Gal in PBS, supplemented with $MgCl_2$ to 20μM immediately before use). After staining, tissues were washed twice with PBS, and then embedded in paraffin or quick frozen for sectioning and histochemical analysis.

EXAMPLE 8

Detection of Functional CFTR Expression in Transfected Cells Using a Chloride Efflux Assay

A chloride ion efflux assay was used to detect functional expression of CFTR in transfected cells.

About 24h prior to introducing CFTR into cells, cells were split into a 6-well tissue culture dish, each well receiving 1mL of 10mL of the cells on the dish plus 3mL media. Cells were returned to the incubator and allowed to grow overnight at 37°C/5% CO_2, or until they were about 70-80% confluent. For assay, media were removed from the wells and each well was washed with 2mL serum-free media. 1mL of serum-free media was then added per well, and the cells incubated at 37°C for 1-2h. 200μl of a DNA-lipid complex comprising a recombinant expression construct encoding CFTR were then added to each well and incubated at 37°C for 6-8h. After this incubation, media were removed from each well, the wells were washed twice with 2mL serum-free media and incubated in 4mL serum-containing media at 37°C for 48h.

The chloride ion efflux assay was performed as follows. Media were aspirated from each of the wells containing cells treated with DNA-lipid complexes, and washed twice with efflux solution (135mM NaCl/2.4mM K_2HPO_4/0.6mM KH_2PO_4/1.2mM

$CaCl_2$/1.2mM $MgCl_2$/10mM glucose/10mM HEPES (pH 7.4)). Cells were then incubated with 1mL efflux solution containing $Na^{36}Cl$ at a final concentration of 2.5μCi/mL $^{36}Cl^-$ for 2h at 37°C. After incubation, the $^{36}Cl^-$-containing efflux solution was aspirated from the cells and the cells then washed each of 4 times with 1mL efflux solution. The cells were then incubated with 1mL efflux solution for 3min at room temperature, and the efflux solution then removed from the cells and transferred into a scintillation vial containing 5mL scintillation cocktail. A fresh aliquot of efflux solution was added to each well and incubated for an additional 3 min. After each incubation, efflux solution was transferred to a scintillation vial containing 5mL scintillation cocktail, and a fresh 1mL aliquot of efflux media was added to the cells and incubated for 3min. These steps of the assay were repeated ten times for a total of 30min. In certain of the wells, $^{36}Cl^-$ ion efflux was stimulated by incubating these cells in the presence of 40μM Forskolin (Sigma), 500μM cpt-cAMP (Sigma), and 100μM IBMX (Sigma) in efflux solution, efflux being stimulated at repetitions 3 through 7.

The extent of $^{36}Cl^-$ ion efflux over this period was determined by scintillation counting, and the basal rate of $^{36}Cl^-$ ion efflux compared with the rate of efflux in cells stimulated by Forskolin/cpt-cAMP/IBMX. Extent of efflux was normalized relative to the amount of $^{36}Cl^-$ ion remaining inside the cells after the 30min incubation. This quantity was determined by lysing the cells by incubating them with 1mL of scintillation fluid for 15min. The lysate from each well was then transferred into a scintillation vial, the well washed with 1mL of efflux solution which was added to the cell lysate, and the $^{36}Cl^-$ ion-associated radioactivity counted.

The results of one such assay are shown in Figure 2. Two plasmids encoding CFTR and differing in the details of the construct (*see* Table I) were tested with (closed circles and boxes) and without (open circles and boxes) stimulation. As is shown in the Figure, stimulation results in the rapid induction of chloride ion efflux over the basal rate of efflux, which efflux persists even after the stimulus is removed (time points 24-30). These results demonstrate the utility of this assay to detect functional expression of CFTR in heterologous cells, and thus forms an *in vitro* standard for determining the vigor of different recombinant expression constructs in expressing human CFTR.

TABLE I

Vectors with the CFTR cDNA

	enhancer	promoter	intron		polyA	antibiotic
MB19 :	HCMV	HCMV	ppi		ppi	amp
MB31:	HCMV	HCMV	ppi		SV40	amp
MB65:	HCMV	HCMV	ppi	Nmyc	ppi	amp
MB66:	HCMV	HCMV	ppi	Cmyc	SV40	amp
MB76:	HCMV	HCMV	ppi		3xSV40	amp
MB77:	---	CC10	ppi		3xSV40	amp
MB78:	HCMV	CC10	ppi		3xSV40	amp
MB81:	----	CFTR	ppi		3xSV40	amp
MB87:	HCMV	CFTR	ppi		3xSV40	amp
MB90:	HCMV	HCMV	---		3XSV40	amp
MB93:	HCMV	HCMV	pgl3		SV40	amp
MB97:	HCMV	HCMV	pgl3		SV40	amp/tet
MB113:	HCMV	HCMV	pgl3		SV40	tet

EXAMPLE 9

Reverse Transcriptase-Polymerase Chain Reaction Analysis

Human CFTR gene expression was assayed using a reverse transcriptase polymerase chain reaction assay (RT-PCR) on transfected tissue culture cells and whole tissues. These assays were performed using vector specific primers and CFTR specific primers. The vector specific primers used were:

5′ AGA TCG CCT GGA GAC GCC AT 3′	forward primer (3651-3671bp in pMB19; Figure 7 and SEQ ID No.:1)

and

5′ GCT CCT AAT GCC AAA GGA AT 3′	reverse primer (1246-1266 bp in pMB19, upstream from hCFTR ATG site; SEQ ID No.:2).

The CFTR specific primers were used:

5′ CCT GTC TCC TGG ACA GAA A 3′	forward primer (3337-3355bp in pMB19; SEQ ID No.: 3)

and

5′ GTC TTT CGG TGA ATG TTC TGA C 3′	reverse primer (3651-3671 bp in pMB19; SEQ ID No.: 4).

Tissues were frozen on dry ice for RT-PCT and stored at -70°C. Tissue samples were homogenized and used directly in this evaluation.

Briefly, RT-PCR was performed by preparing first-strand cDNA from cellular RNA isolated from frozen tissues using standard techniques (*see* Sambrook *et al.*, *ibid.*), including specifically the use of random hexamer for priming and MMLV-derived reverse transcriptase. cDNA was used in PCR reactions performed as follows. The entire 25μL of the first-strand cDNA reaction was mixed with the components of the PCR reaction (under standard conditions; *see* Innis *et al.*, 1990, PCR Protocols: A Guide to Methods and Applications, Academic Press, New York), including 25μM apiece of each of the specific pairs of PCR primers. PCR reactions were overlayed with light mineral oil to prevent condensation and then subjected to the following PCR

cycling protocol:

1 cycle	10min 94°C
30 cycles	1min 94°C
	2min 55°C
	3min 72°C
1 cycle	10min 72°C
	2min 27°C.

After completion of the reaction, the apparatus was programmed to take and hold the reaction mixtures at 4°C until analysis.

PCR products were analyzed by electrophoresis in agarose or acrylamide gels. In these assays, the vector-specific primers were expected to yield a band representative of plasmid DNA (485bp) and a hCFTR RNA-specific band (142bp). The CFTR-specific primers were expected to yield a DNA fragment band of 334bp.

EXAMPLE 10

Functional Delivery of CAT Gene Constructs to Cells *In Vivo*

Functional delivery of a variety of CAT reporter gene constructs was achieved using different embodiments of the DNA:lipid complexes of the invention.

A. DOTIM:Cholesterol Formulation I

DOTIM:cholesterol liposomes were prepared as described above in 1:1 ratio and used to prepare DNA:lipid complexes. DOTIM:cholesterol (1:1) liposomes were used to make DNA complexes using the chloramphenicol acetyl transferase (CAT) expression vector p4119 (Figure 3). DNA:lipid complexes were prepared having a DNA:lipid ratio of 1:6, and using 125μg of DNA per 200μL complex. Liposome size was determined by optical density (OD) at 400 nm. A total of 200μL of the complex were injected into the tail veins of 3 ICR mice. At 24 hrs post-injection, tissues were harvested and prepared for CAT assays as described in Example 4 above. Tissues harvested included lung, liver, kidney, spleen, ovary, brain, smooth muscle, heart and ear.

Results of these CAT assays are shown in Tables II and III below. Table II shows CAT activity as total ^{14}C-labeled chloramphenicol counts converted to acetyl and diacetyl forms by CAT expression vector-encoded enzyme activity in lung for each of the three experimental animals tested.

TABLE II

animal number	CAT (cpm)
20.1-1	800,000
20.1-2	1,400,000
20.1-3	400,000

Table III shows CAT assay data from a variety of tissues from one of the experimental animals (animal 20.1-2). These results demonstrate that intravenous inoculation of mice in the tail vein with DOTIM:cholesterol:DNA complexes in this formulation results in preferential targeting of the DNA:lipid complexes to the lungs, with CAT activity in lung tissue representing over 80% of the CAT activity detected in all mouse tissues tested.

TABLE III

tissue	CAT (cpm)
lu	1,400,000
li	20,000
sp	63,000
ki	15,000
ov	3,000
br	7,000
sm	58,000
he	115,000
ear	1500

Key: lung (lu), liver (li), kidney (ki), spleen (sp), ovary (ov), brain (br), smooth muscle (sm), heart (he).

The results of these experiments are also shown graphically in Figure 4, which summarizes the results obtained with over 700 experimental and control mice. As can be seen in the Figure, treated mice reproducibly showed greater than 1000-fold higher CAT activity in lung of mice treated with the DNA:lipid complexes of the invention comprising CAT-encoding recombinant expression constructs (a total of 555 mice), compared with control (untreated) mice (a total of 163 mice).

The delivery and uptake into cells of various mouse tissues of the CAT plasmid DNA administered as DNA:lipid complexes of the invention by injection into the tail vein of mice was analyzed by Southern blot analysis using routine procedures (*see* Sambrook *et al. ibid.*). DNA from mouse tissues was extracted, purified and digested with *Bam*HI restriction endonuclease. The resulting DNA restriction fragments were separated by agarose gel electrophoresis and transferred to a membrane by capillary action. Such membranes were dried, prehybridized and then hybridized with a radioactively-labeled, CAT DNA-specific probe (about 10^8-10^9 dpm/μg) at an appropriate stringency (2X-6X SSC at 62°C) overnight, washed to high stringency (0.1-0.5X SSC at 65°C) and exposed to autoradiographic film at -70°C using intensifying screens.

Results of these experiments are shown in Figure 5. The lower panel is identical to the upper panel, but has been allowed to expose the X-ray film for a longer period of time. These results demonstrate that CAT DNA is introduced specifically into lung, with significant amounts of DNA uptake in spleen. Much lower amounts of CAT DNA were observed in certain other tissues (liver, kidney) but many tissues showed essentially no CAT-specific hybridization, even at the longer exposure time.

Lung tissue from untreated animals was analyzed to determine the specific cell types transfected. Histological sections were analyzed for vector-specific mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR), performed as described in Example 9. The results shown in Figure 21 indicated that expression of the transgene was predominantly found in vascular endothelial cells.

A second series of experiments were performed using this lipid formulation. In these experiments, the DNA construct used was the β-galactosidase expression vector MB10 (*see* Table I) that encodes a form of β-galactosidase that is translocated

into the nucleus in *in vitro* studies. Complexes were formed as described above, and mice were injected with 200μL complexes in the tail vein. The resulting β-galactosidase levels present in lungs are shown in Figure 6, which represents the results of experiments with 9 experimental and 1 control (administered liposome only) mouse.

In a third series of experiments, expression of the human CFTR gene was shown following IV delivery of DNA/DOTIM:cholesterol complexes. A recombinant expression plasmid encoding the human CFTR gene (MB19; *see* Table I and Figure 7) was used to make DNA:lipid complexes as described above (DNA/lipid ratio of 1:6, 125μg DNA/200μL complex). These complexes were tested by transfection/chloride ion efflux assay in human 293 cells *in vitro*, as described in Example 8, and 200μL was injected into each of ICR 3 mice. Cells and lungs were harvested at 24 hrs. RNA was made using conventional methods as embodied in kits from either Stratagene (for cell culture results) or 5'-3' Prime (for lung tissues). Samples were analyzed by RT-PCR as described above in Example 9. In this analysis, amplification of plasmid sequences yielded a 484bp PCR product, while amplification of cDNA corresponding to spliced CFTR mRNA for CFTR yielded a 142bp PCR product. Similar results were obtained from lungs following IV administration of the CFTR/lipid complexes.

The time course of expression of exogenously added CAT-encoding plasmid in mouse lung was determined. A number of mice were injected intravenously in the tail vein with DNA/lipid complexes comprising p4119 CAT DNA at a 1:6 ratio with DOTIM:cholesterol at a concentration of 125μg/200μl. Mice were sacrificed in duplicate over a period of 55 days, and lung tissue analyzed by CAT assay as described above. These results are shown in Figure 8, which indicated that high-level, persistent expression of the reporter gene construct had been achieved..

Complexes of this DOTIM:DNA formulation were also administered by direct intracranial delivery. Complexes were made using CAT expression plasmid p4119 and complexed with DOTIM:cholesterol (1:1) at a ratio of 1:1 DNA:lipid at a DNA concentration of 500μg/200μL. 200μL of these complexes were directly implanted intracranially, and the extent of CAT activity is brain tissue analyzed 24h later. The

results of these experiments are shown Figure 9.

The results of these different assays indicated that this DOTIM:cholesterol formulation was capable of delivering a variety of recombinant expression constructs to the lung after intravenous administration, as well as by direct injection into a tissue of interest (brain).

B. DOTIM:Cholesterol Formulation II

DOTIM:cholesterol liposomes were prepared as described above in 1:1 ratio and used to prepare DNA:lipid complexes. DOTIM:cholesterol (1:1) liposomes were used to make DNA complexes using the chloramphenicol acetyl transferase (CAT) expression vector p4119. DNA:lipid complexes were prepared having a DNA:lipid ratio of 1:1, and using 200-550μg of DNA per 200μL complex. Liposomes were injected into the tail vein of ICR mice, as described above.

CAT gene expression in lung tissue from mice injected with DOTIM:cholesterol:DNA complexes prepared at a DNA/lipid ratio of 1:1 was determined. Plasmid p4119 DNA was complexed with DOTIM:cholesterol formulation of the invention, the complexes having a DNA/lipid ratio of 1:1. Tail vein injections were performed and tissues harvested at 24 hrs as described.

The results of these assays are shown in Table IV below.

TABLE IV

Amount of DNA/complex	OD_{400}*	lung expression**
200μg/200μl	0.24	39,000
300μg/200μl	0.32	9,000
400μg/200μl	0.53	500,000
500μg/200μl	0.66	700,000
550μg/200μl	0.82	1,000,000
negative control	0.03	0

* light scattering as an estimate of complex size

** in cpm of acetylated and diacetylated ^{14}C-labeled chloramphenicol

DNA/DOTIM complexes were made using plasmid p4119 and DNA/DOTIM:cholesterol liposomes at ratios of 1:6 and 1:8, were held at 40°C for 11 days prior to testing and then tested again at 18 days. The results of CAT expression assays using these formulations are shown in Table V.

TABLE V

DNA/DOTIM ratio	time stored	CAT/lung**
1:6	11 days	515,000
1:8	11 days	1,050,000
1:6	18 days	11,000,000
1:8	18 days	3,450,000

** in cpm of acetylated and diacetylated ^{14}C-labeled chloramphenicol

DOTIM:cholesterol complexes with DNA were also administered by intraperitoneal injection. DNA:liposome complexes administered intravenously and intraperitoneally were compared, using CAT expression plasmid p4119 DNA complexed with DOTIM:cholesterol (1:1) formulations of the invention. In these assays, the complexes had a DNA/lipid ratio of 1:1 and a DNA concentration of 300-500μg/200μL. A total of 1mL of these complexes was injected intraperitoneally in two mice (mice 4 and 5), 200μL were administered intravenously (mice 1 and 2), and 1 mouse (mouse 3) was administered a formulation comprising only liposomes. Tissues were harvested at 48h post-injection. CAT assays were performed as described above in Example 3, and the results of these assays are shown in Figure 10 for heart (he), spleen (sp), pancreas (pa) and lung (lu).

The effect on the efficiency of DNA delivery to tissues *in vivo* of intravenously administering different formulations comprising the same mixture of cationic and neutral lipids was determined by comparing the extent of transferred CAT activity observed using the different formulations. CAT plasmid DNA/DOTIM:DOPE (1:1) complexes were prepared in the following formulations:

A. DNA:Lipid ratio of 1:6 DNA concentration of 0.625mg/mL

B. DNA:Lipid ratio of 1:1 DNA concentration of 2.5mg/mL

Each formulation was prepared as described in Examples 1 and 3 above, and were administered by intravenous injection into the tail vein of cohorts of 3 ICR mice per tested formulation. Liposomes that were not complexed with DNA were injected into a separate cohort of 3 mice as a control.

Animals were sacrificed 1-2 days after injection and analyzed by CAT assay of spleen, heart and lung tissue. The results of these experiments are shown in Figure 11. This Figure demonstrates that Formulation B provides a consistently higher level of CAT activity in spleen, heart and lung than Formulation A, although it appears that the relative efficiency of plasmid delivery is about the same for both formulations.

C. Comparison of HLA Gene Delivery using Different DNA:Lipid Complexes

Three different lipid formulations were used to deliver a human HLA-encoding construct to bone marrow, spleen and lymph node. The three formulations used were:

A. DOTIM:cholesterol (1:1) DNA:lipid ratio 1:6 DNA concentration 0.625mg/mL
B. DOTIM:cholesterol (1:1) DNA:lipid ratio 1:1 DNA concentration 2mg/mL
C. DOTIM:DOPE (1:1) DNA:lipid ratio 1:1 DNA concentration 2mg/mL

(DOPE is dioleoylphosphatidylethanolamine). Each formulation was prepared as described in Examples 1 and 3 above, and were administered by intravenous injection into the tail vein of cohorts of 3 ICR mice per tested formulation. Liposomes that were not complexed with DNA were injected into a separate cohort of 3 mice as a control.

Animals were sacrificed 1-2 days after injection and analyzed by histochemical staining for human HLA expression. Tissues were analyzed for percentage of cells in the tissue positive for human HLA expression in the histochemical staining assay. Results of these experiments are shown in Figure 12, wherein Formulation A is MB102, Formulation B is MB107 and Formulation C is MB163. For each formulation tested, some cells in each of the tissues were found to stain positive for human HLA expression. Lymph node staining varies most among different administered formulations, with DOTIM:cholesterol at the higher (2mg/mL) DNA concentration providing the most human HLA positive cells, and the DOTIM:DOPE formulation providing the least human HLA positive cells. The results in spleen were

less variable, with the DOTIM:cholesterol formulation at the lower (0.625mg/mL) DNA concentration providing the most human HLA positive cells. Bone marrow cells showed high levels of human HLA positive cells with all formulations tested.

In view of these results, a series of experiments were performed to demonstrate formulation-dependent targeting of DNA:lipid complexes to spleen and lung. Two formulations were used:

a. DOTIM:cholesterol (1:1) DNA:lipid ratio 1:6 DNA concentration 0.625mg/mL
b. DOTIM:DOPE (1:1) DNA:lipid ratio 1:1 DNA concentration 1.5mg/mL

Each formulation was prepared as described in Examples 1 and 3 above, using a CAT-encoding construct, and were administered by intravenous injection into the tail vein of cohorts of 3 ICR mice per tested formulation. Liposomes that were not complexed with DNA were injected into a separate cohort of 3 mice as a control.

Animals were sacrificed 1-2 days after injection and analyzed by CAT assay of lung and spleen tissues as described above. The results of these experiments are shown in Figure 13, wherein MB102 is Formulation A and MB153 is Formulation B. CAT activity is expressed as the percentage of total ^{14}C-chloramphenicol counts converted to acetylated and diacylated forms associated with each tissue. As can be seen from the Figure, the DOTIM:cholesterol formulation administered intravenously resulted in 96% (of over 1 million counts) being localized to lung tissue; 2% of the counts resulting from this formulation were found in the spleen, and the rest were found in other tissues. In contrast, the DOTIM:DOPE formulation administered intravenously resulted in 91% (of 160,000 counts) being localized to spleen tissue, with about 3% of the counts being found in the lung and the rest being found in other tissues. These results demonstrate that this DOTIM:cholesterol formulation specifically targets the DNA:lipid complex to the lung, while the DOTIM:DOPE formulation specifically targets DNA:lipid complexes to the spleen. In addition, these results show that CAT activity is about 10-fold more robust when delivered in DOTIM:cholesterol complexes to the lung than the CAT activity resulting from DOTIM:DOPE complex-mediated delivery to spleen.

D. Intraperitoneal Delivery Formulations

Liposome formulations were developed for targeted gene delivery by intraperitoneal administration. DOTIM:cholesterol formulations (1:1) were tested using a CAT-encoding construct at a DNA:lipid ratio of 1:1 and a total DNA concentration in the complex of 2.5mg/mL. An amount (1mL) of these DNA complexes were injected into the peritoneal cavity of each of 4 mice; an equal volume of the liposome formulation not complexed with CAT-encoding DNA was injected into 4 mice in a separate cohort as a control. Peritoneal macrophages were isolated 24-48h after injection and tested for CAT activity as described above.

The results of these experiments are shown in Figure 14. Peritoneal macrophages from control (untreated) mice showed essentially no CAT activity in this assay. Macrophages from mice administered the DNA:lipid complexes in this formulation intraperitoneally showed high levels of CAT activity, demonstrating specific *in vivo* delivery of a functional CAT gene using this formulation.

Spleens from these animals were also tested and CAT activity compared to peritoneal macrophages. These results are shown in Figure 15, where it can be seen that CAT activity in macrophages was much higher than in spleen, demonstrating specificity in targeting to these cells.

Pancreatic tissues were targeted for gene delivery using the DNA:lipid formulations of the invention as follows. A formulation comprising a CAT-expressing plasmid and DOTIM:DOPE (1:1), at a DNA:lipid ratio of 1:1, and a total DNA concentration in the complex of 1.5mg/mL was injected intraperitoneally into a cohort of 3 mice. Two mice were injected with the liposome formulation not complexed with DNA as a control. Pancreas and lung tissues were analyzed 24-48h post-administration for CAT activity as described above.

The results of these experiments are shown in Figure 16. These results demonstrate that this formulation specifically targets delivery of CAT-encoding DNA constructs to the pancreas when administered intraperitoneally.

A CAT-encoding recombinant construct was targeted to spleen using yet another DNA:lipid formulation. Plasmid DNA that was complexed with DOTIM:cholesterol (1:1), at a DNA:lipid ratio of 1:1, and a total DNA concentration in the complex of 2.5mg/mL was injected intraperitoneally into a cohort of 3 mice.

A separate cohort of mice were injected with the liposome formulation not complexed with DNA as a control. Spleen tissues from mice in each cohort were analyzed 24-48h post-administration for CAT activity as described above.

The results of these experiments are shown in Figure 17. These results demonstrate that this formulation specifically targets delivery of CAT-encoding DNA constructs to spleen *in vivo* when administered intraperitoneally.

The tissue specificity of intraperitoneal delivery was demonstrated by comparison of two different formulations administered intraperitoneally. The following formulations were tested:

a. DOTIM:cholesterol (1:1) DNA:lipid ratio 1:1 DNA concentration 2mg/mL
b. DOTIM:DOPE (1:1) DNA:lipid ratio 1:1 DNA concentration 2mg/mL

Each formulation was prepared as described in Examples 1 and 3 above, using a CAT-encoding construct, and were administered by intraperitoneal injection of cohorts of 3 ICR mice per tested formulation. Liposomes that were not complexed with DNA were injected into a separate cohort of 3 mice as a control.

Animals were sacrificed 1-2 days after injection and analyzed by CAT assay of pancreas and spleen tissues as described above. The results of these experiments are shown in Figure 18, where MB153 is Formulation A, and MB152 is Formulation B. CAT activity is expressed as the percentage of total ^{14}C-chloramphenicol counts converted to acetylated and diacylated forms associated with each tissue. As can be seen from the Figure, the DOTIM:DOPE formulation administered intraperitoneally resulted in 96% (of 18 million counts) being localized to pancreatic tissue; 3% of the counts resulting from this formulation were found in the spleen, and the rest were found in other tissues. In contrast, the DOTIM:cholesterol formulation administered intraperitoneally resulted in 58% (of 28 million counts) being localized to spleen tissue, with about 42% of the counts being found in the pancreas; essentially no CAT activity was observed in other tissues. These results demonstrate that this DOTIM:DOPE formulation specifically targets the DNA:lipid complex to the pancreas when administered intraperitoneally, while the DOTIM:cholesterol formulation specifically targets DNA:lipid complexes to the pancreas and spleen.

E. Direct Delivery Formulations

Liposome formulations were developed for targeted gene delivery by direct injection into tissues. DOTIM:cholesterol formulations (1:1) were tested using a CAT-encoding construct at a DNA:lipid ratio of 1:1 and a total DNA concentration in the complex of 2.5mg/mL. This formulation was directly injected in 1.5mL into a human prostate *ex corpora*, and then assayed by CAT assay as described above.

The results of this experiment are shown in Figure 19. This Figure illustrates the resilts of four different prostate tissues tested, detected as the amount of CAT activity found in each of the transfected prostate tissues.

These results demonstrate that gene delivery can be mediated by direct injection of DNA:lipid complexes on the invention into human tissues.

F. Comparison of Intravenous and Intraperitoneal Administration Routes

The effect of administration route on targeted delivery of CAT-encoding plasmid DNA using a single DNA:lipid complex formulation was determined. DOTIM:cholesterol formulations (1:1) were tested using a CAT-encoding construct at a DNA:lipid ratio of 1:1 and a total DNA concentration in the complex of 2.5mg/mL. Cohorts of 3 mice were either injected intravenously in the tail vein, or intraperitoneally. Spleen and lung tissues were analyzed 24-48h post-administration for CAT activity as described above. The results of these experiments are shown in Figure 20. It can be seen from the Figure that the highest CAT activity levels were achieved in lung tissue following intravenous administration of the formulation. However, CAT activity after intraperitoneal administration was relatively higher in spleen than in lung. These results demonstrate that tissue-specific targeting of DNA delivery can be achieved with the same efficacious formulation of DNA:lipid complexes, and that the targeted site can be influenced by the route of administration.

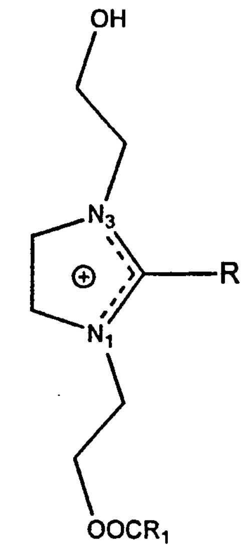
It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

WE CLAIM:

1. A pharmaceutical composition comprising a formulation of a soluble complex of a recombinant expression construct and a mixture of a neutral lipid and a cationic lipid in a pharmaceutically acceptable carrier suitable for administration to an animal by intravenous, intraperitoneal or direct injection into the animal, wherein

(a) the recombinant expression construct comprises a nucleic acid encoding a transcription product and wherein said nucleic acid is operatively linked to gene expression regulatory elements whereby the nucleic acid is transcribed *in vivo*; and

(b) the cationic lipid is a compound having formula I:



where R and R_1 are independently straight-chain, aliphatic hydrocarbyl groups of from 11 to 29 carbon atoms.

2. The pharmaceutical composition of Claim 1 wherein the cationic lipid is 1-(2-(oleoyloxy)ethyl)-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride.

3. The pharmaceutical composition of Claim 1 wherein the neutral lipid is cholesterol or dioleoylphosphatidylethanolamine.

4. The pharmaceutical composition of Claim 1 wherein the cationic lipid is 1-(2-(oleoyloxy)ethyl)-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride and the

neutral lipid is cholesterol or dioleoylphosphatidylethanolamine.

5. The pharmaceutical composition of Claim 4 wherein the cationic lipid and the neutral lipid are present in a molar ratio of about 1:1.

6. The pharmaceutical composition of Claim 1 wherein the complex of a recombinant expression construct and a mixture of a neutral lipid and a cationic lipid comprises a ratio of DNA to cationic lipid of about 1:6 to about 1:15 μg/mL.

7. The pharmaceutical composition of Claim 1 wherein the complex of a recombinant expression construct and a mixture of a neutral lipid and a cationic lipid comprises a ratio of DNA to cationic lipid of about 1:1.

8. The pharmaceutical composition of Claim 1 wherein the nucleic acid comprising the recombinant expression construct is present in the formulation at a concentration of about 0.5mg/mL to about 5mg/mL.

9. Use of a pharmaceutical composition of Claim 1 to prepare a medicament for introducing a recombinant expression construct into a cell comprising lung tissue in an animal wherein the pharmaceutical composition is administered to the animal by intravenous injection.

10. A use according to Claim 9 wherein the cationic lipid is 1-(2-(oleoyloxy)ethyl)-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride, the neutral lipid is cholesterol, the cationic lipid and the neutral lipid are present in a molar ratio of 1:1, the complex of a recombinant expression construct and a mixture of a neutral lipid and a cationic lipid comprises a ratio of DNA to cationic lipid of 1:6, and the DNA concentration in the DNA:lipid formulations is 0.625mg/mL.

11. Use of a pharmaceutical composition of Claim 1 to prepare a medicament for introducing a recombinant expression construct into a cell comprising

spleen tissue in an animal wherein the pharmaceutical composition is administered to the animal by intravenous injection.

12. A use according to Claim 11 wherein the cationic lipid is 1-(2-(oleoyloxy)ethyl)-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride, the neutral lipid is dioleoylphosphatidylethanolamine or cholesterol, the cationic lipid and the neutral lipid are present in a molar ratio of 1:1, the complex of a recombinant expression construct and a mixture of a neutral lipid and a cationic lipid comprises a ratio of DNA to cationic lipid of 1:1, and the DNA concentration in the DNA:lipid formulations is about 1-2.5mg/mL.

13. Use of a pharmaceutical composition of Claim 1 to prepare a medicament for introducing a recombinant expression construct into a cell comprising a peritoneal macrophage in an animal wherein the pharmaceutical composition is administered to the animal by intraperitoneal injection.

14. A use according to Claim 13 wherein the cationic lipid is 1-(2-(oleoyloxy)ethyl)-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride, the neutral lipid is cholesterol, the cationic lipid and the neutral lipid are present in a molar ratio of about 1:1, the complex of a recombinant expression construct and a mixture of a neutral lipid and a cationic lipid comprises a ratio of DNA to cationic lipid of about 1:1, the DNA concentration in the DNA:lipid formulations is about 1-2.5mg/mL, and the tissue is spleen.

15. A use according to Claim 13 wherein the cationic lipid is 1-(2-(oleoyloxy)ethyl)-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride, the neutral lipid is dioleoylphosphatidylethanolamine, the cationic lipid and the neutral lipid are present in a molar ratio of about 1:1, the complex of a recombinant expression construct and a mixture of a neutral lipid and a cationic lipid comprises a ratio of DNA to cationic lipid of about 1:1, the DNA concentration in the DNA:lipid formulations is about 1.5-2.5mg/mL, and the tissue is pancreas.

16. A use according to Claim 13 wherein the cationic lipid is 1-(2-(oleoyloxy)ethyl)-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride, the neutral lipid is cholesterol, the cationic lipid and the neutral lipid are present in a molar ratio of about 1:1, the complex of a recombinant expression construct and a mixture of a neutral lipid and a cationic lipid comprises a ratio of DNA to cationic lipid of about 1:1, the DNA concentration in the DNA:lipid formulations is about 1-2.5mg/mL, and the tissue is peritoneal macrophage.

17. Use of a pharmaceutical composition of Claim 1 to prepare a medicament for introducing a recombinant expression construct into a cell in an animal wherein the pharmaceutical composition is administered to the animal by direct injection.

18. A use according to Claim 17 wherein the cationic lipid is 1-(2-(oleoyloxy)ethyl)-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride, the neutral lipid is cholesterol, the cationic lipid and the neutral lipid are present in a molar ratio of about 1:1, the complex of a recombinant expression construct and a mixture of a neutral lipid and a cationic lipid comprises a ratio of DNA to cationic lipid of about 1:1, and the DNA concentration in the DNA:lipid formulations is about 1-2.5mg/mL.

19. Use of a pharmaceutical composition of Claim 1 to prepare a medicament for introducing a recombinant expression construct into a cell comprising brain tissue in an animal wherein the pharmaceutical composition is administered to the animal by direct intracranial injection.

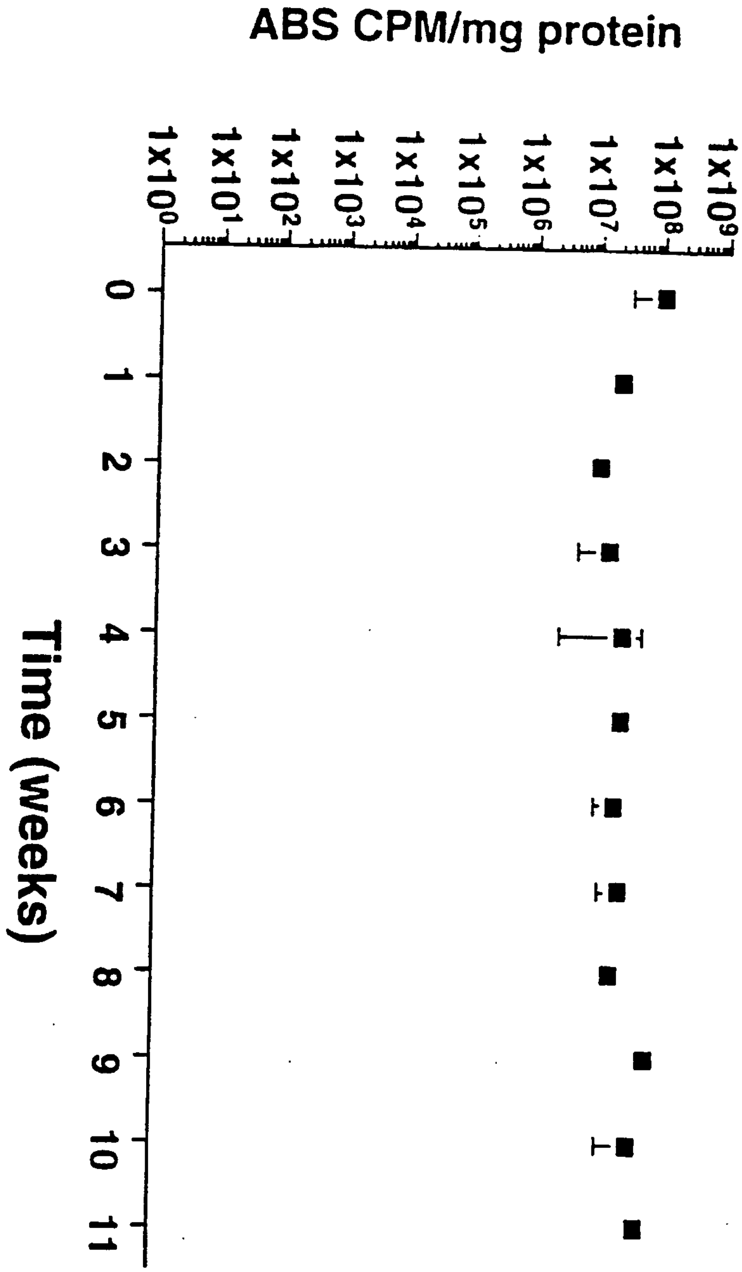


Figure 1

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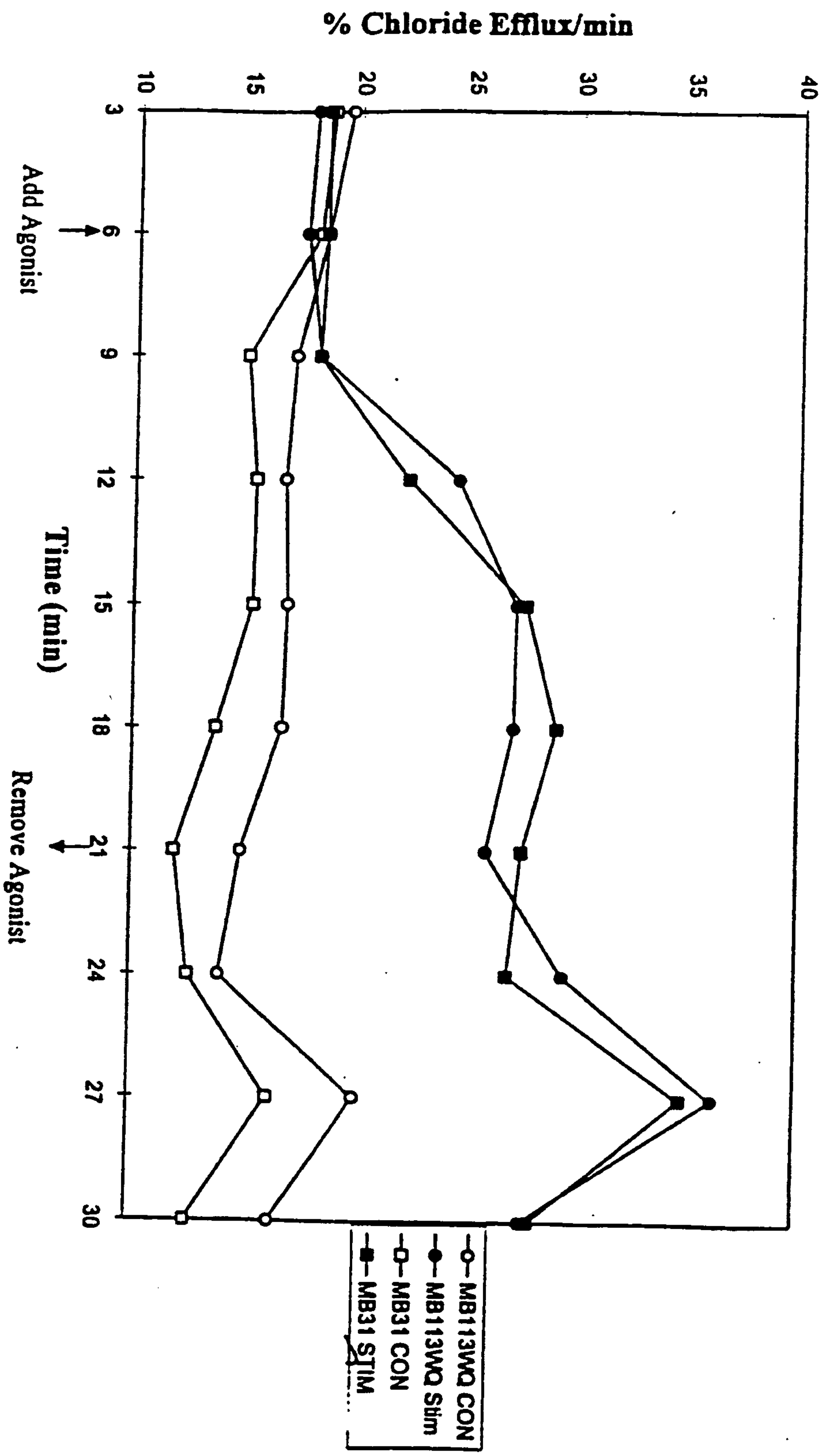

Figure 2

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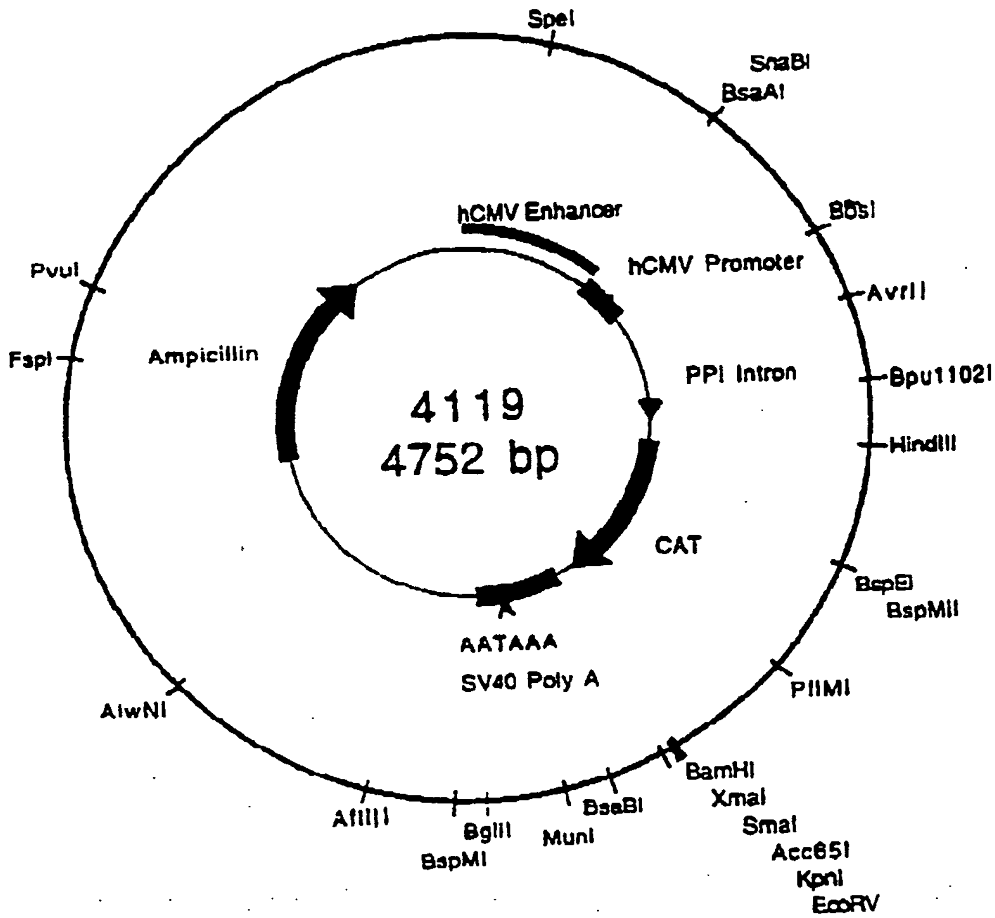

Figure 3

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Figure 4

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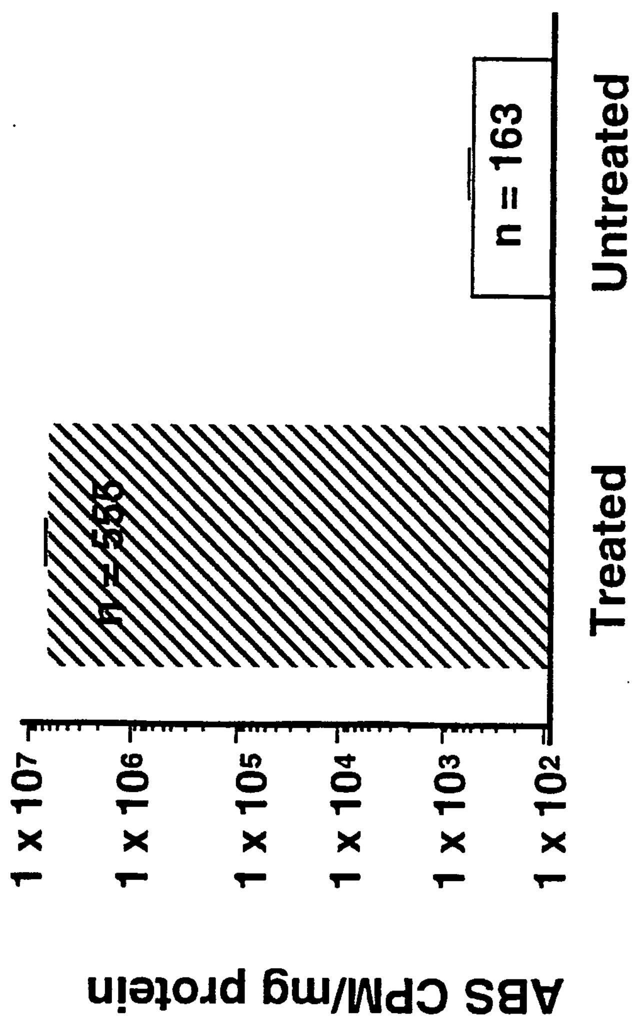

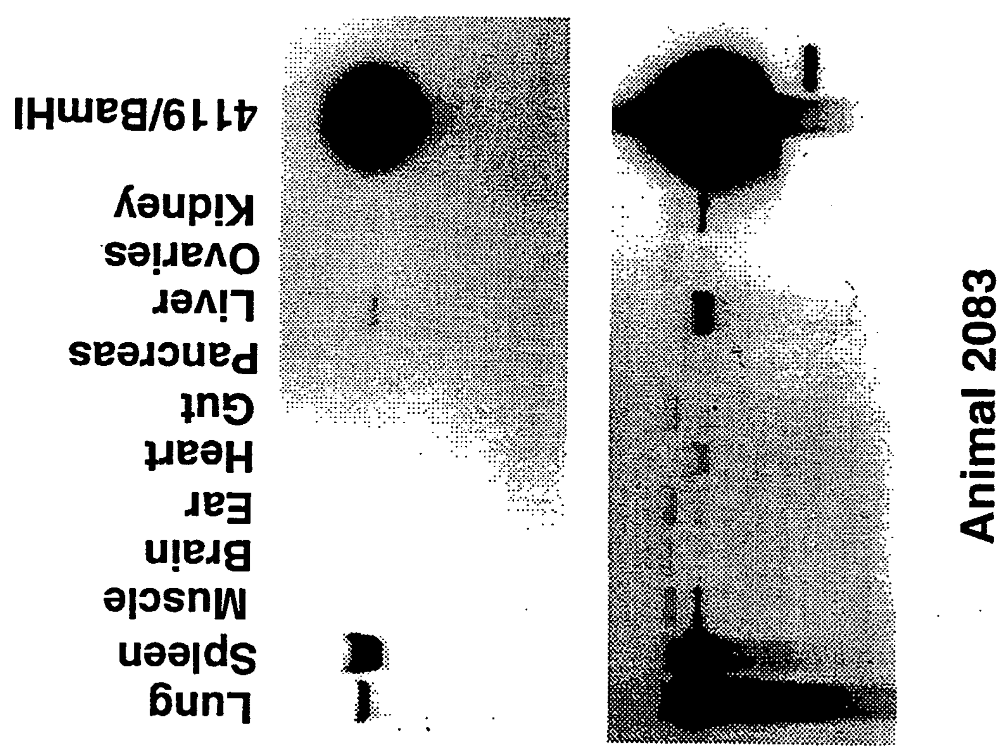

Figure 5

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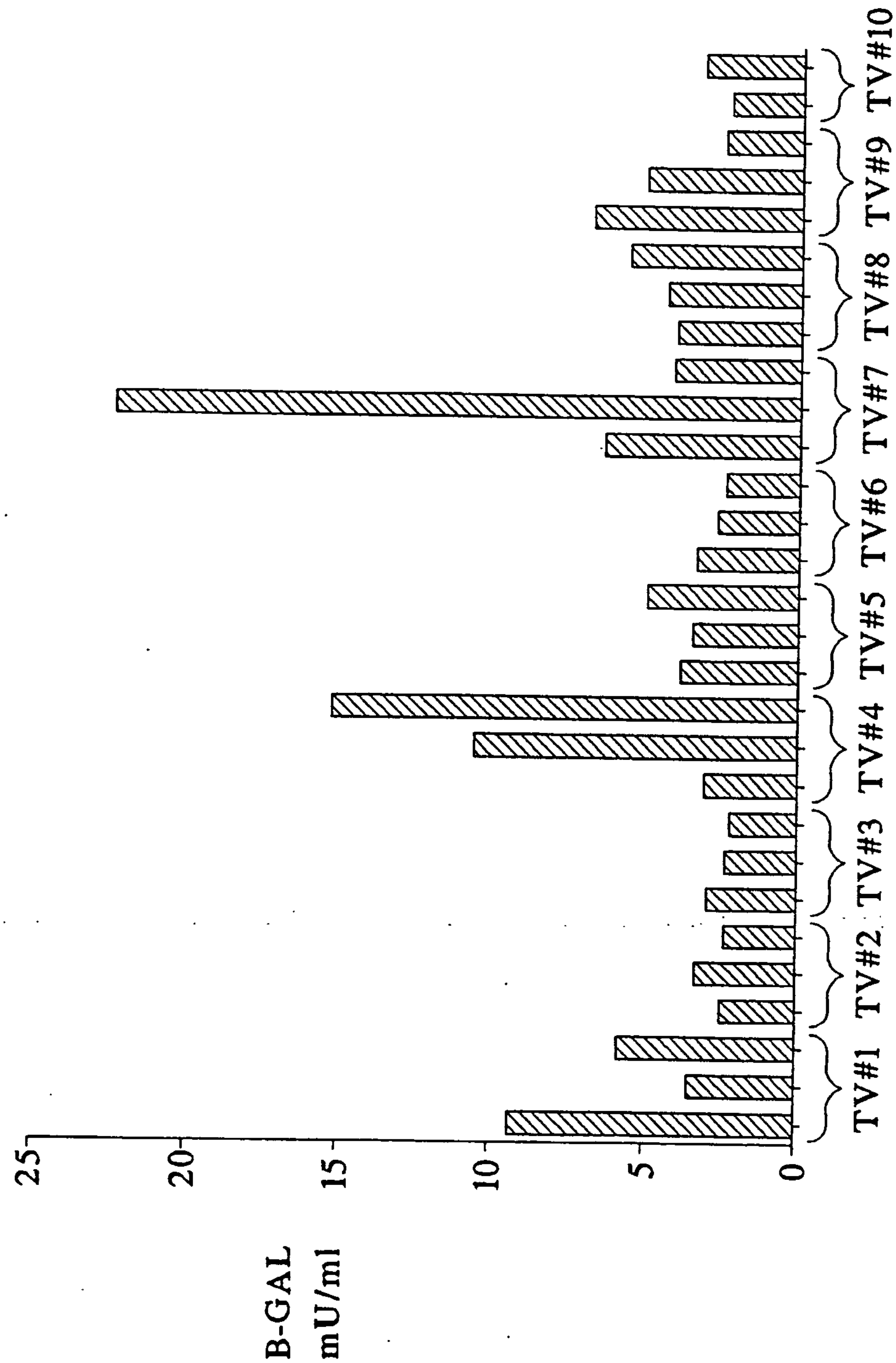
FIG. 6




HCMV Enhancer + Promoter
Cap Site
ATG CFTR
Intron
cDNA
pMB19
8577 bp
AmpR
Poly A
End CFTR

Figure 7

Figure 8

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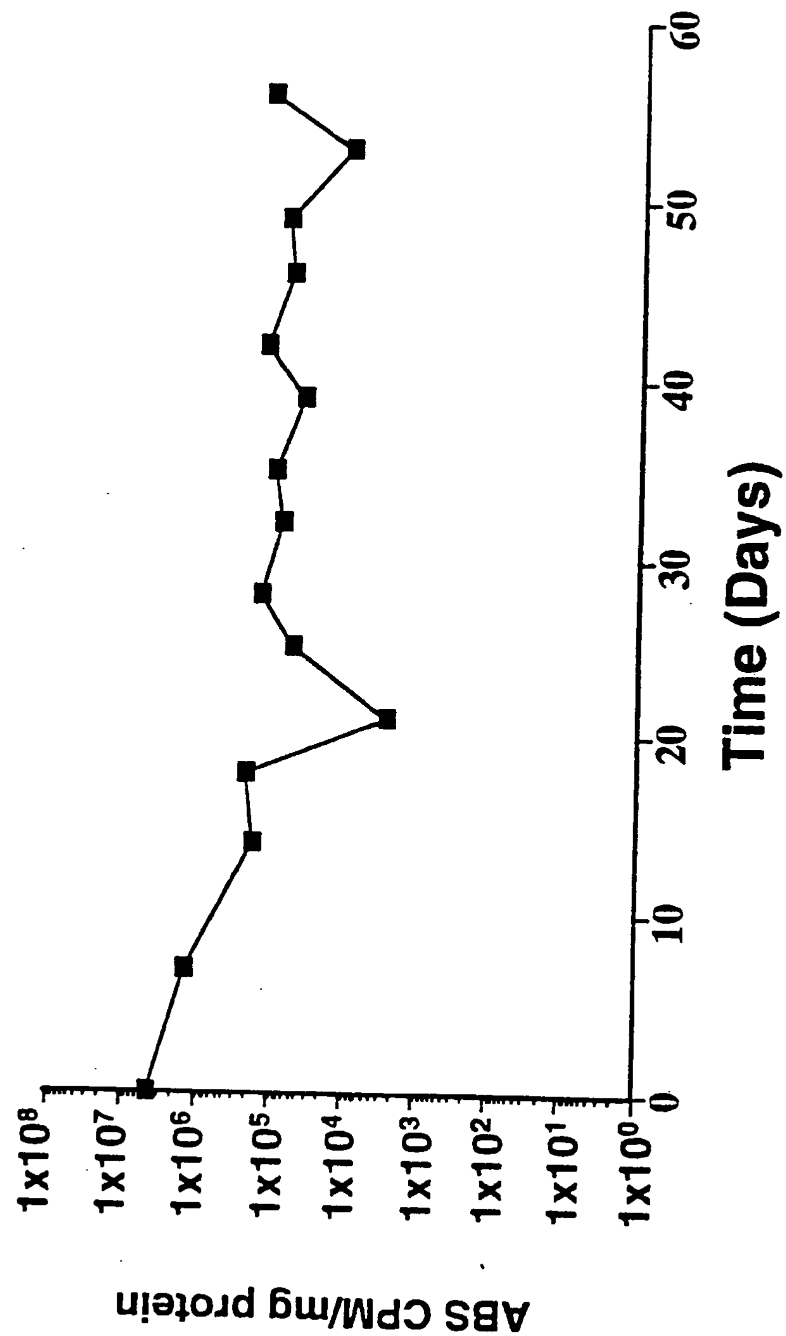

Figure 9

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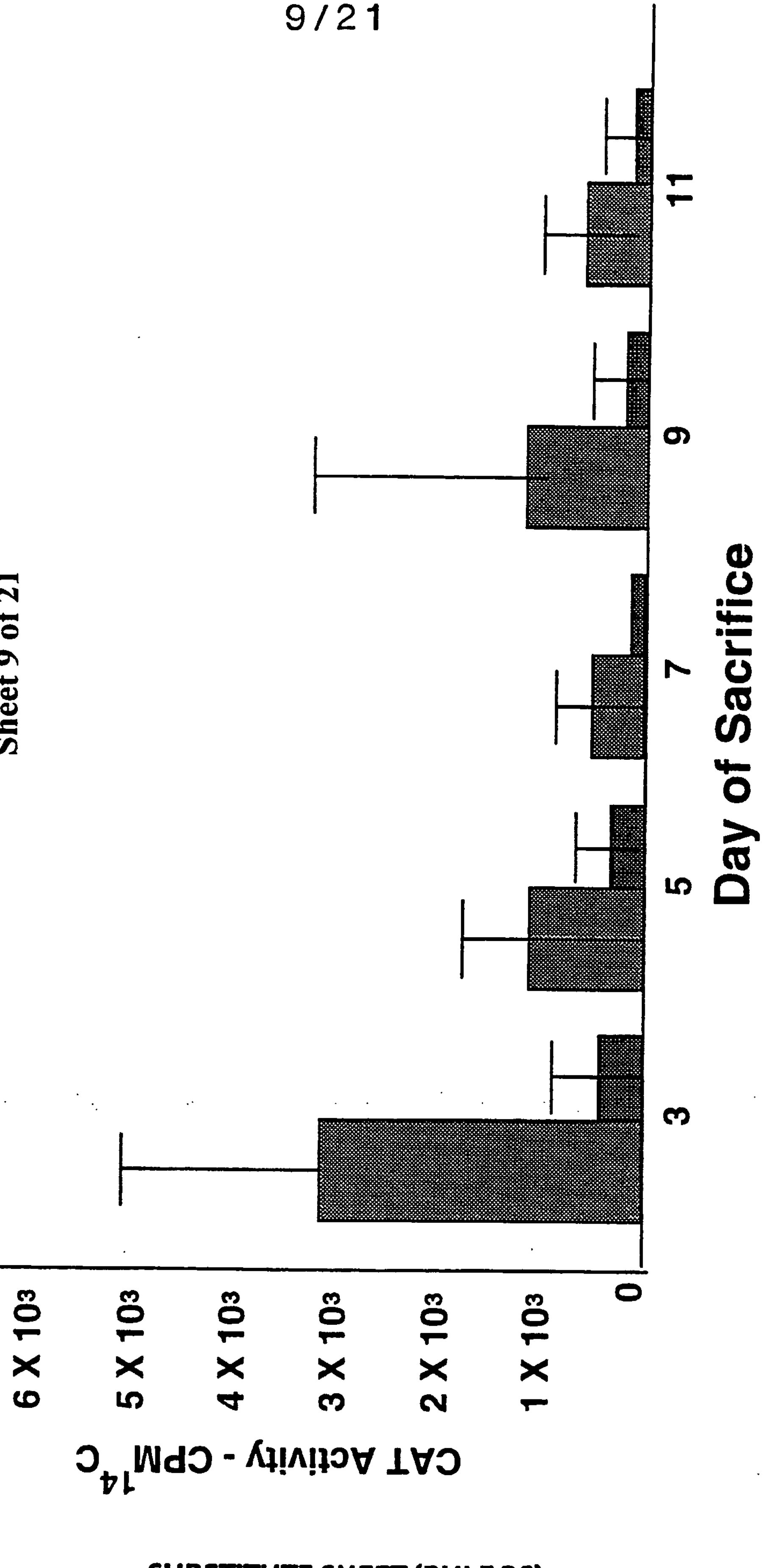

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FIG. 10

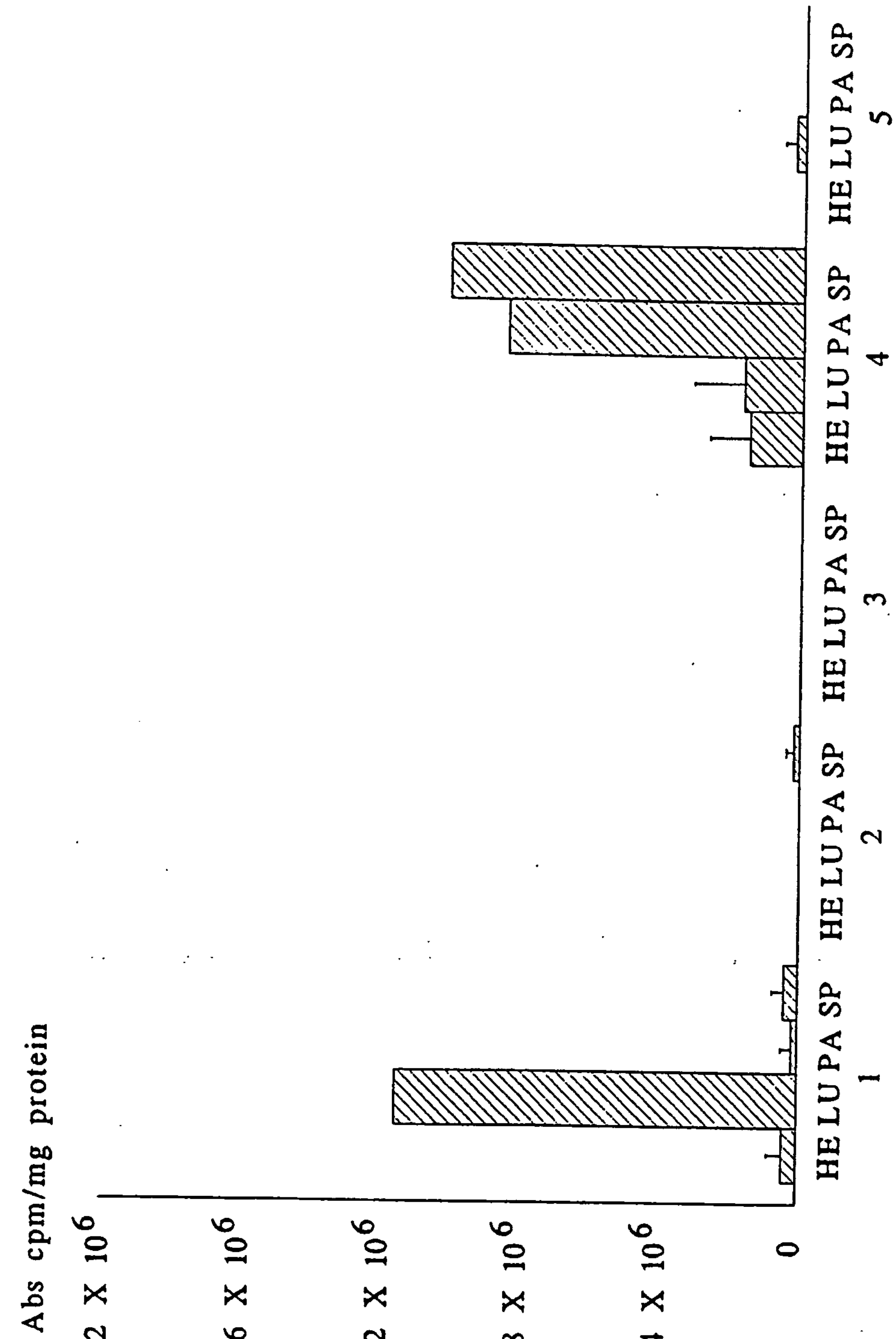

Figure 11

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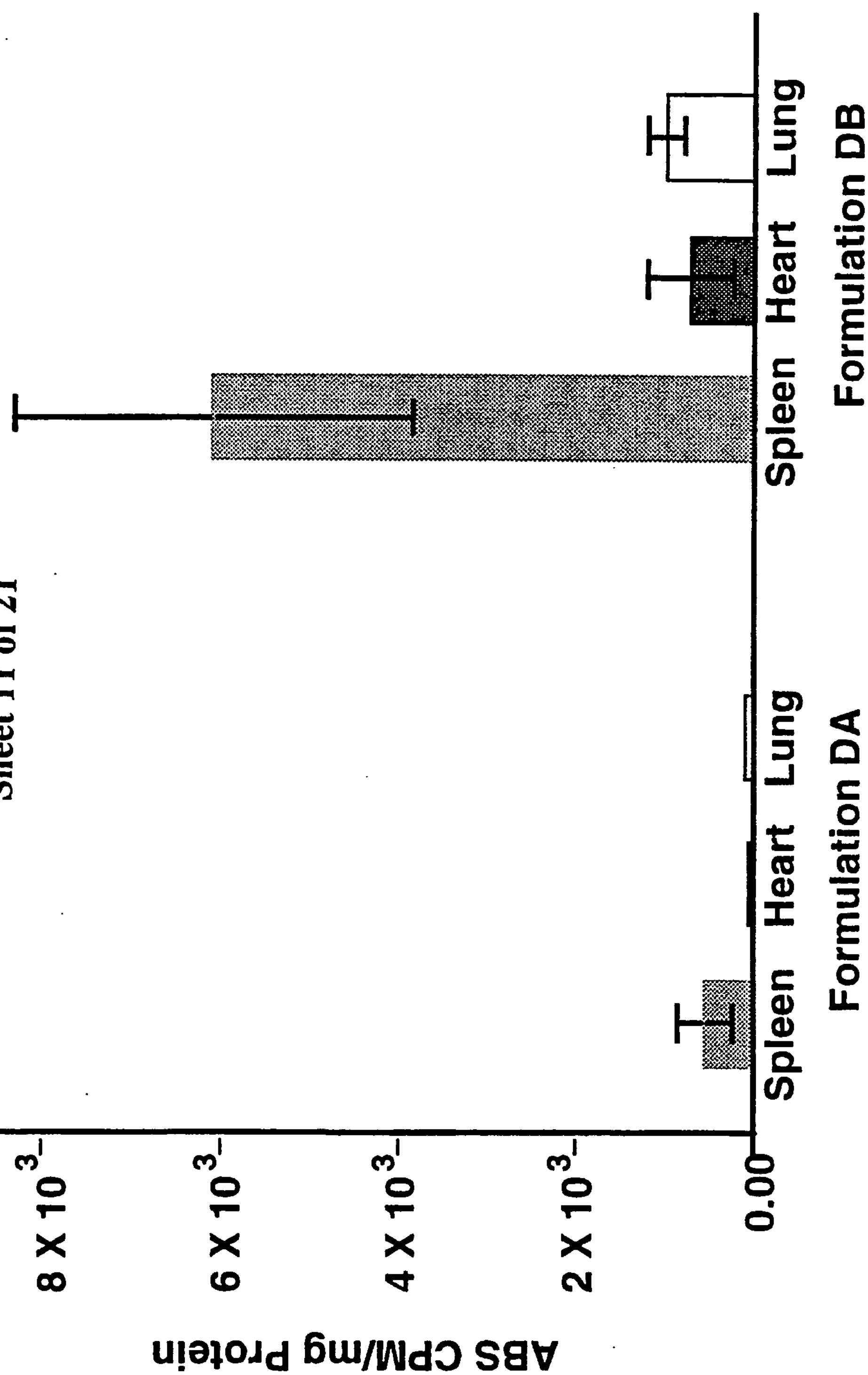

Figure 12

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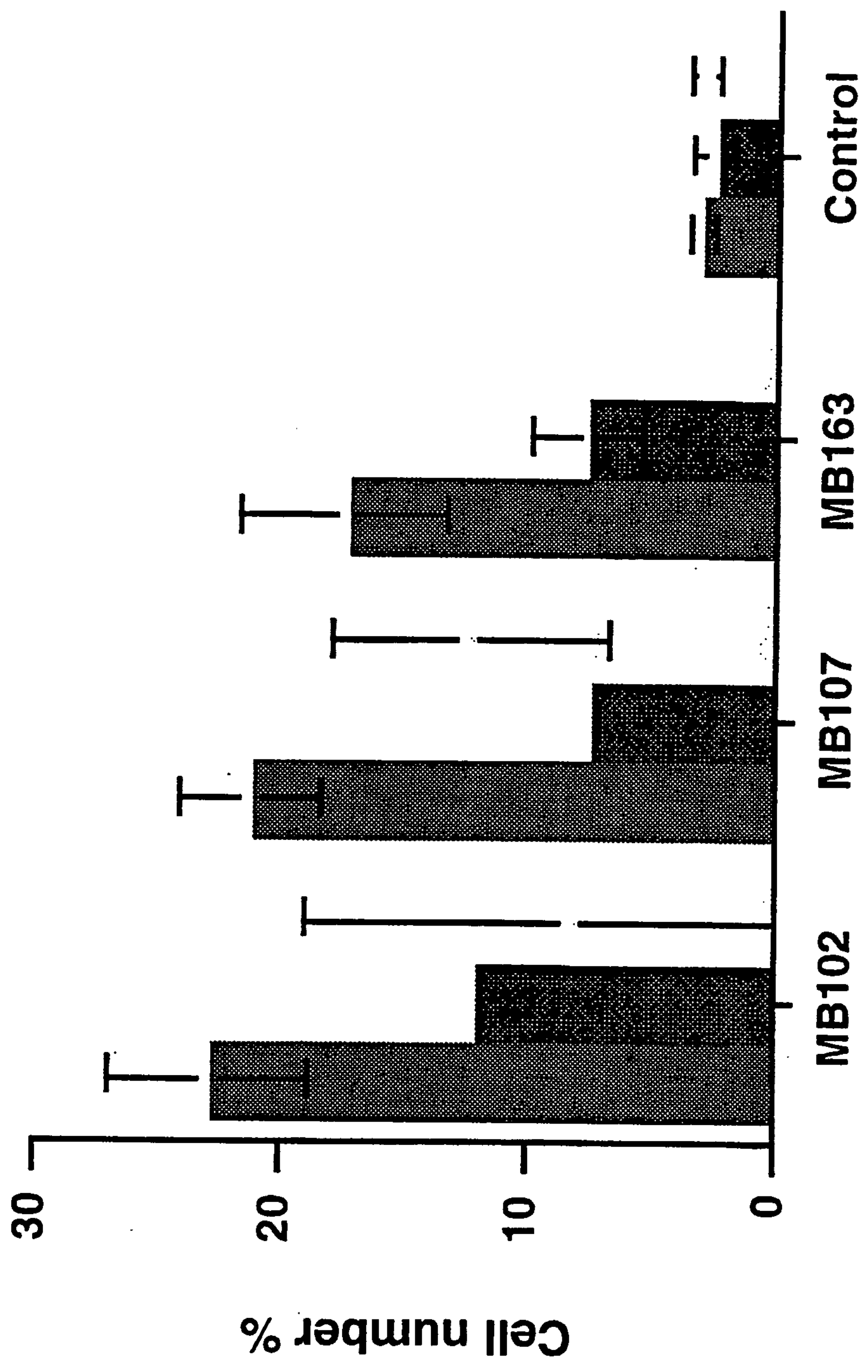

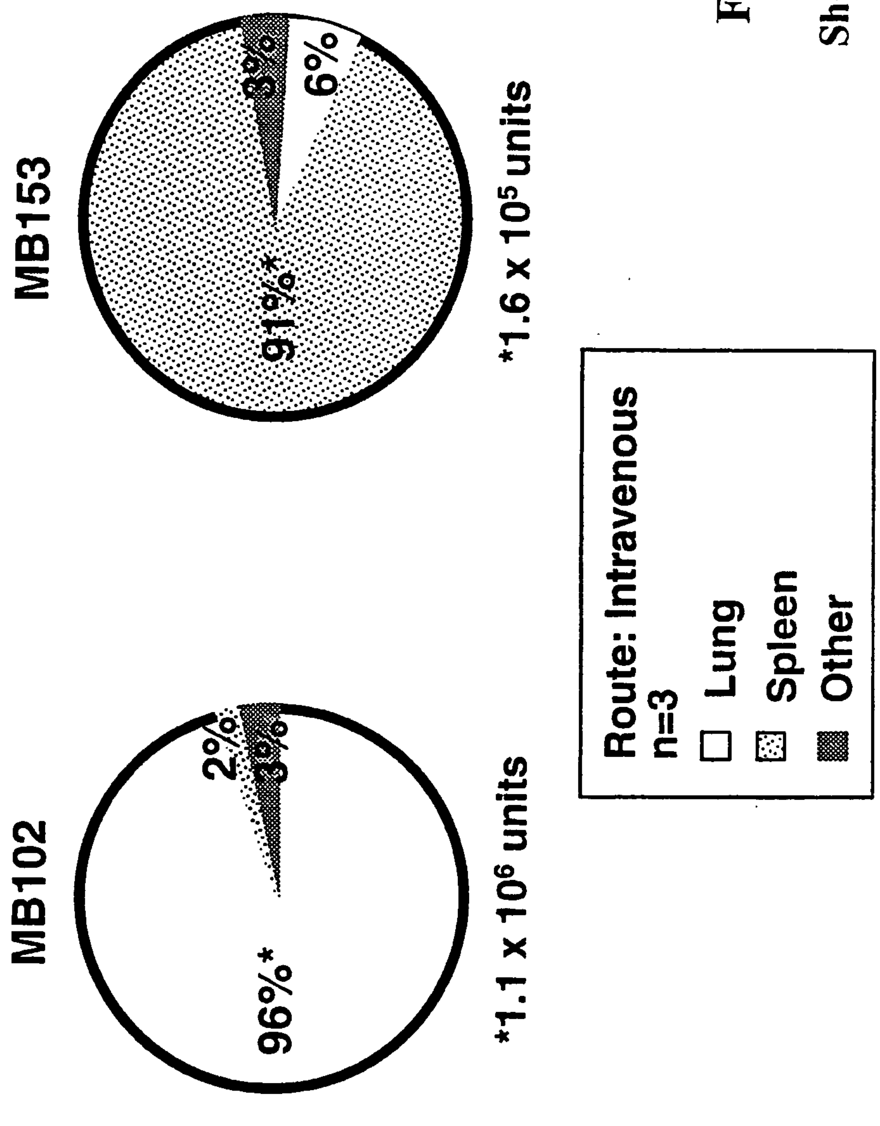

Figure 13

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Figure 14

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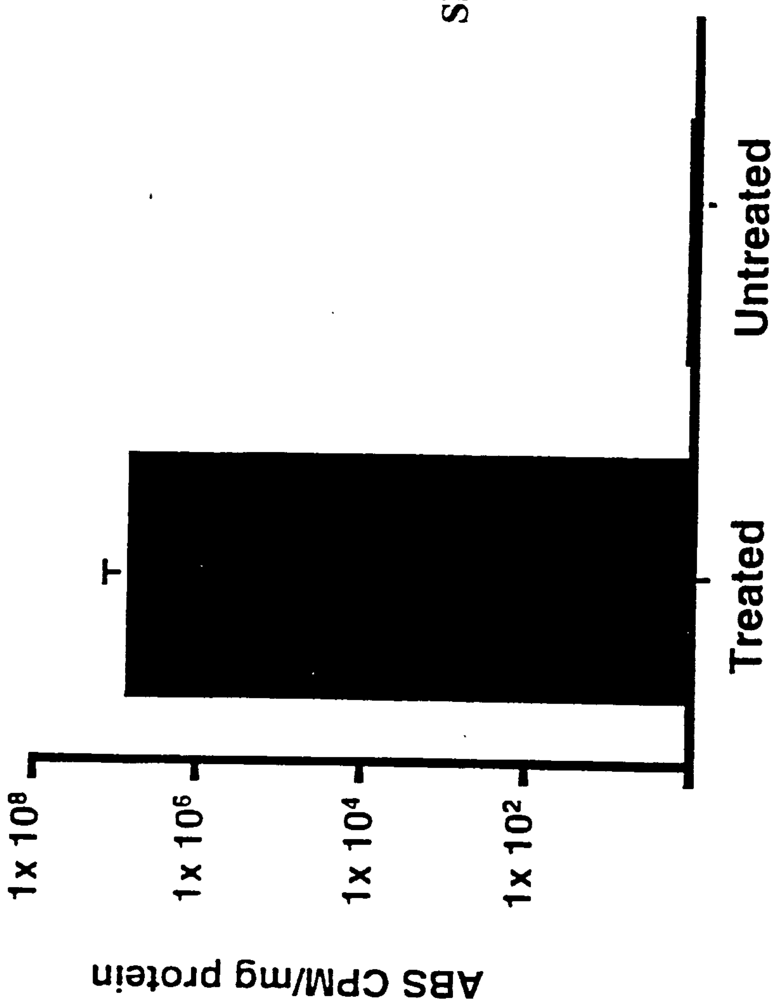

FIG. 15

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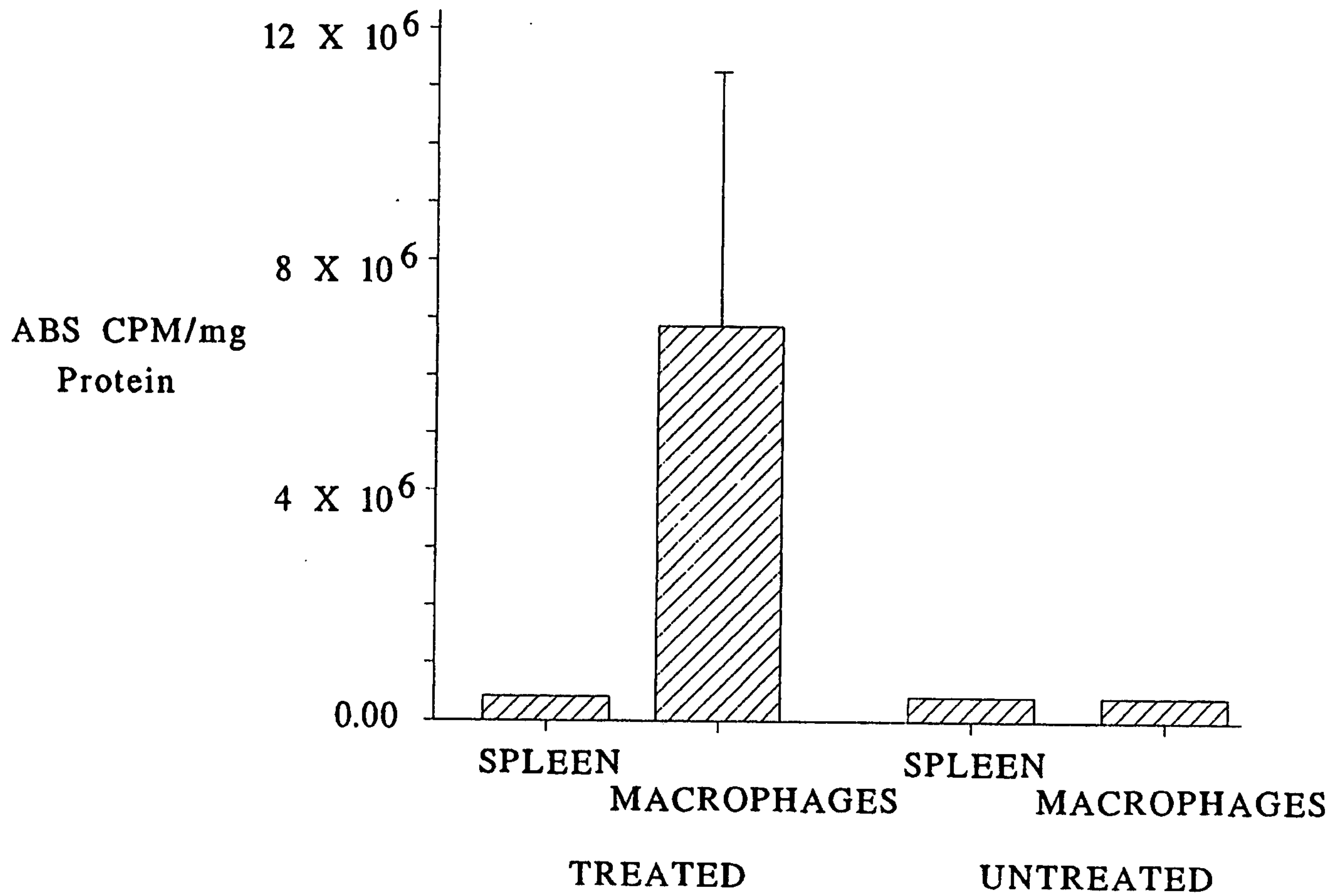

FIG. 16

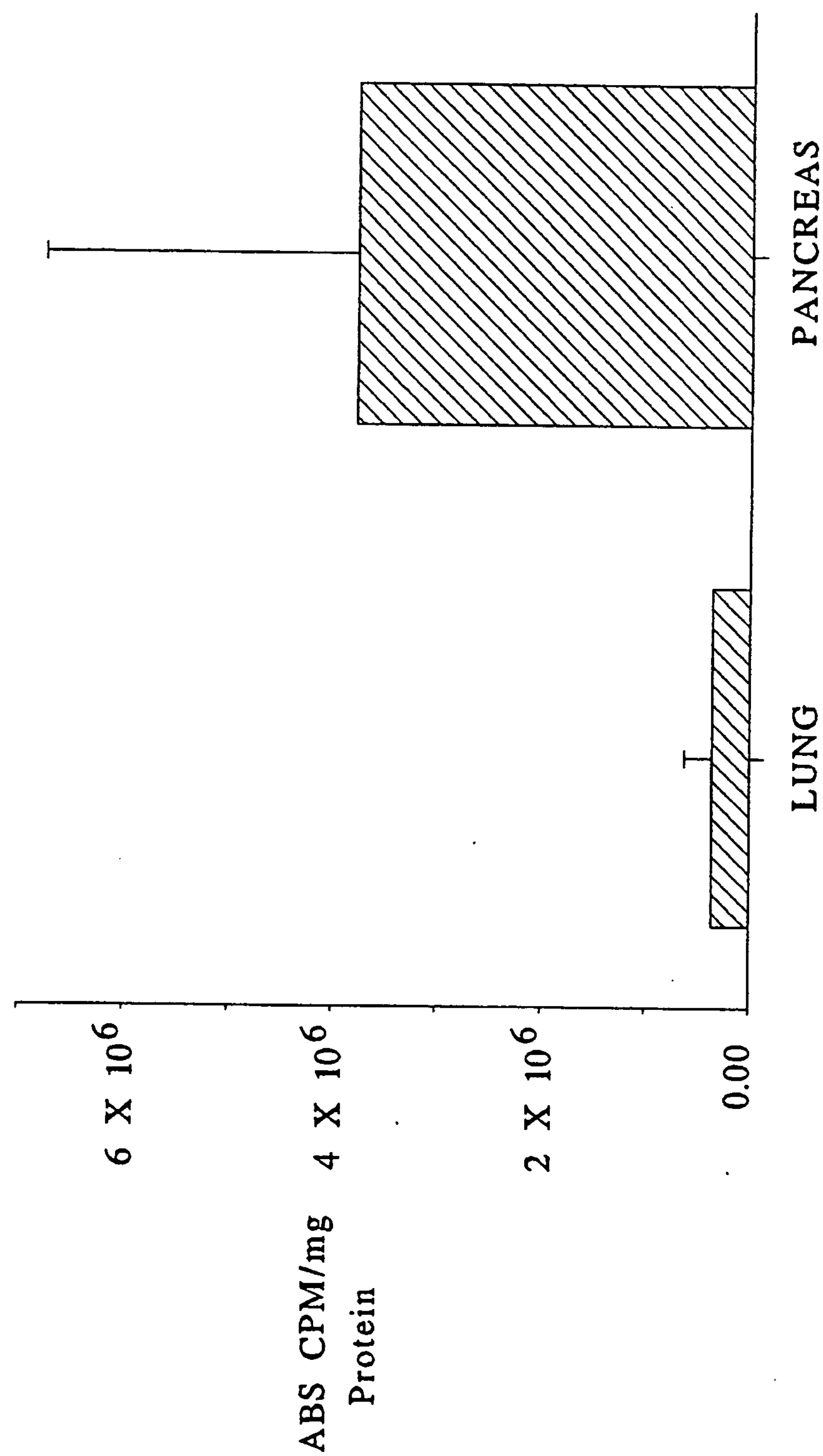

Figure 17

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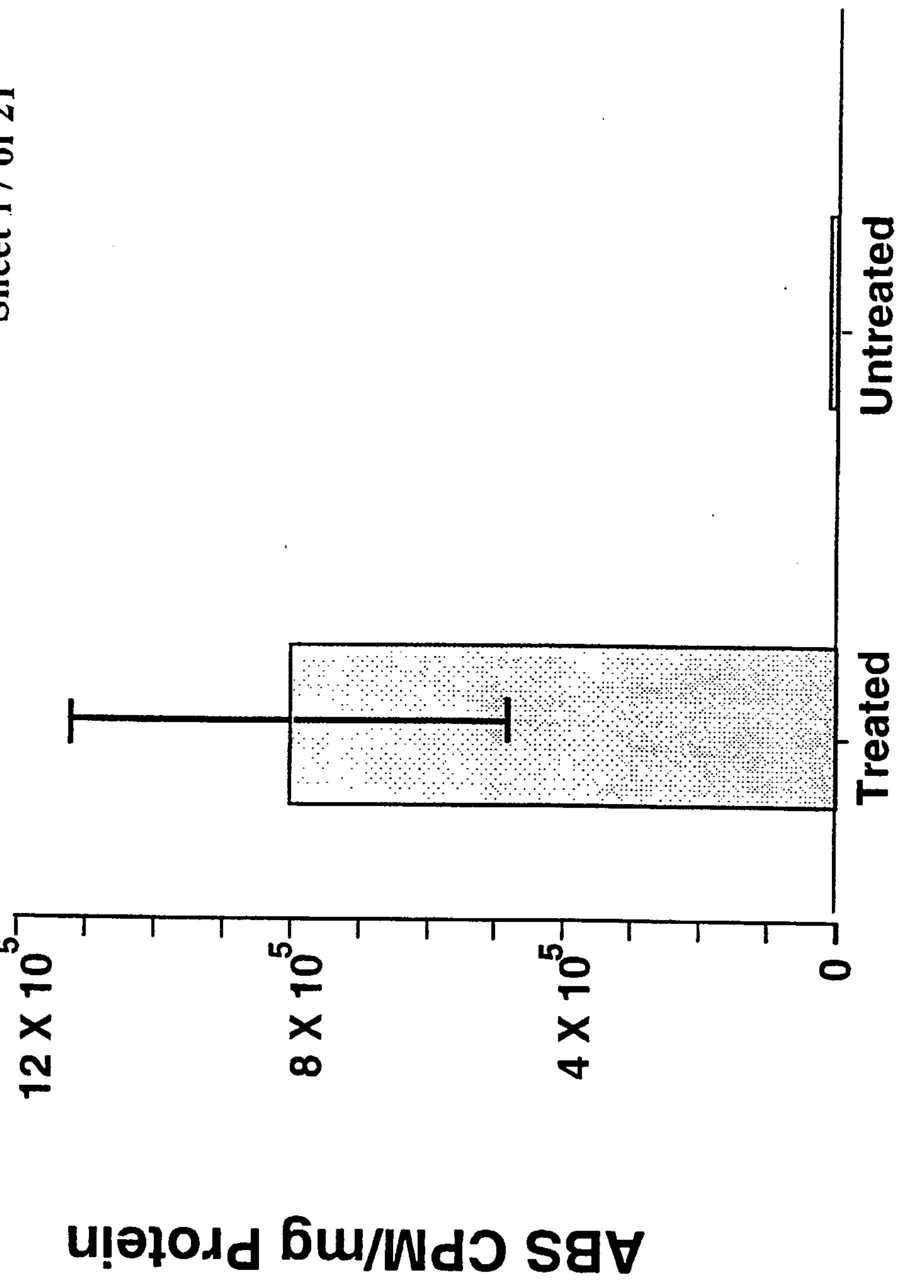

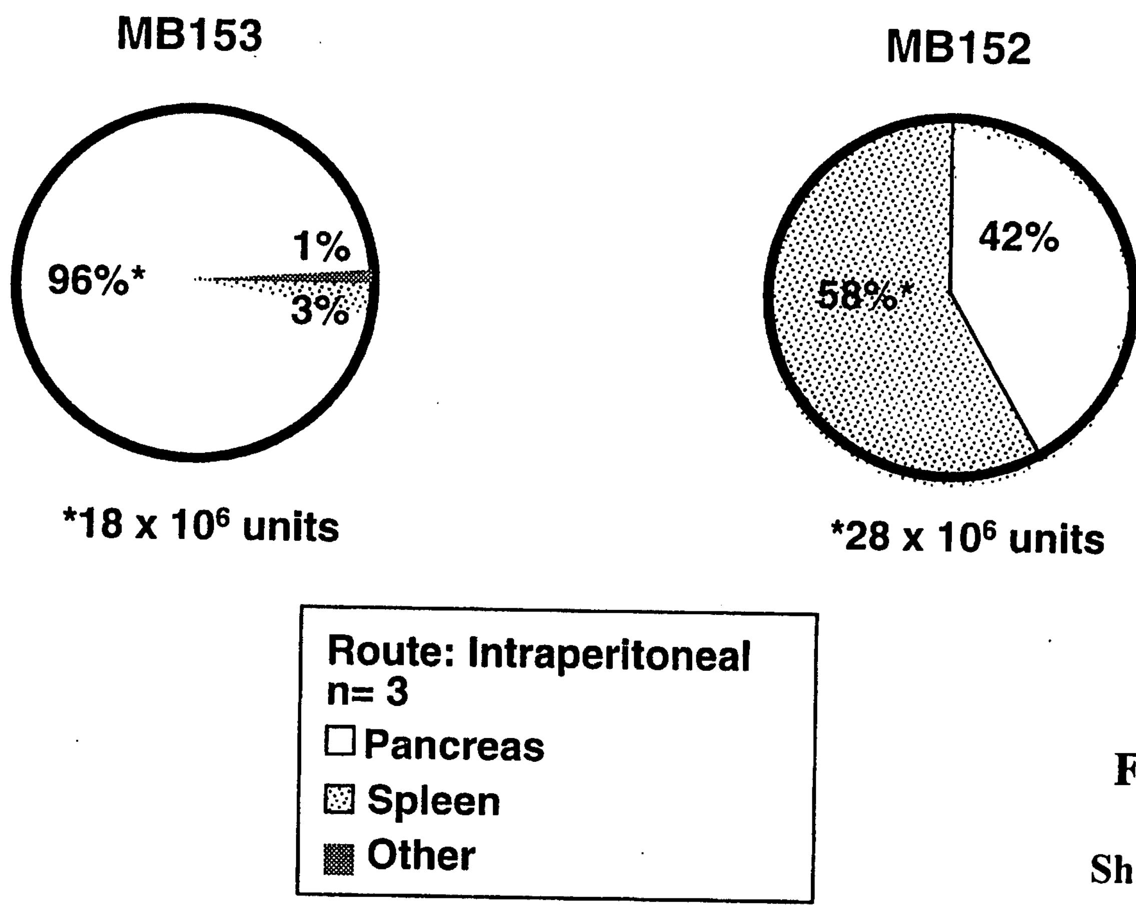
MB153
96%*
1%
3%
*18 x 10^6 units
MB152
42%
58%*
*28 x 10^6 units
Route: Intraperitoneal
n= 3
Pancreas
Spleen
Other

Figure 18

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FIG. 19

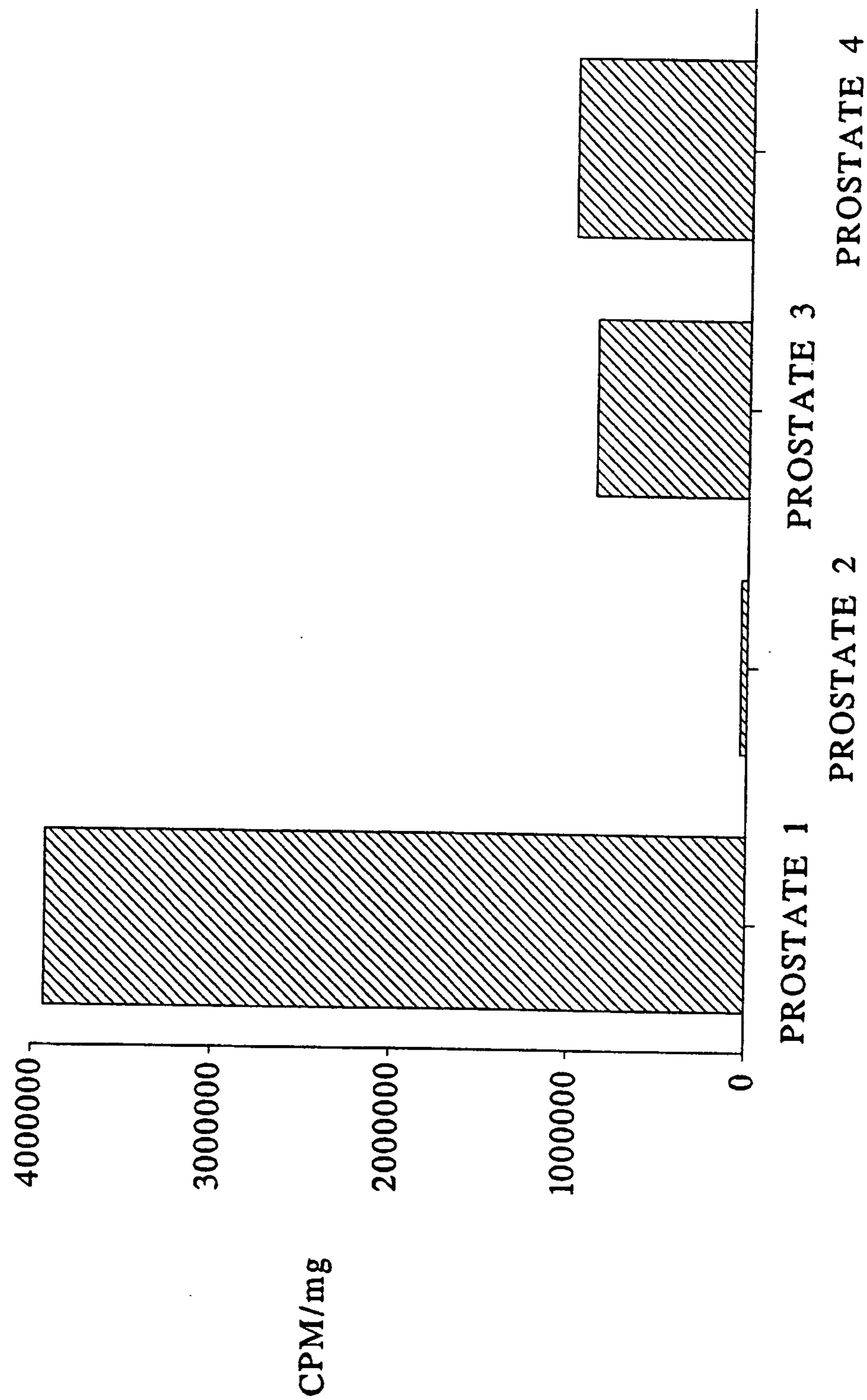

Figure 20

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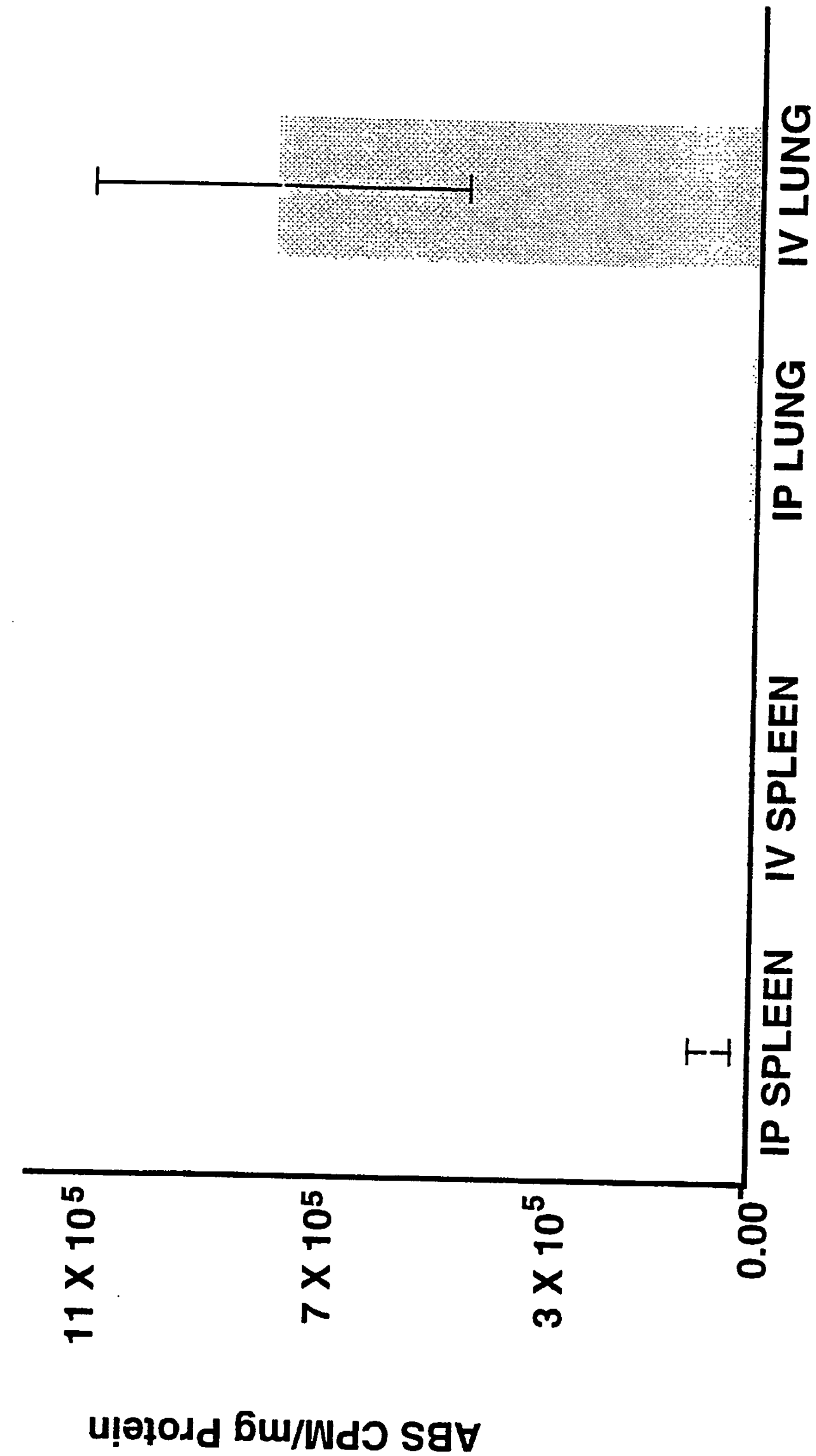

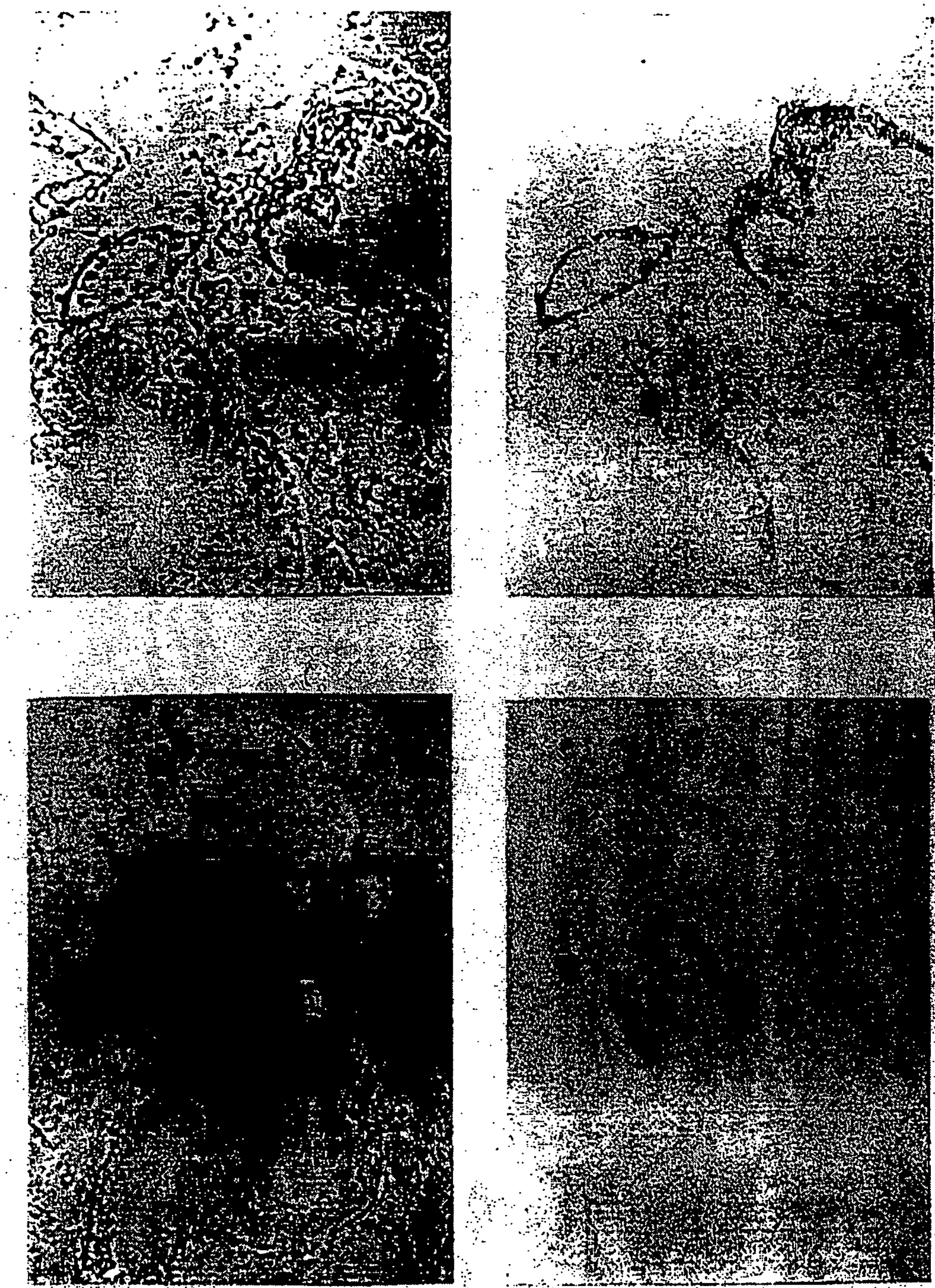

CAT Transfected Animals

Untreated

Figure 21

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/09526

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/88 A61K47/48 A61K9/127 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,95 14380 (MEGABIOS CORPORATION) 1 June 1995 see the whole document ---	1-9, 11-14, 16-18
T	BIOCHEMISTRY, vol. 34, no. 41, 17 October 1995, pages 13537-13544, XP000602216 SOLODIN ET AL.: "A novel series of amphiphilic imidazolinium compounds for in-vitro and in-vivo gene delivery" see the whole document ---	1-8
A	WO,A,94 26915 (THE REGENTS O F THE UNIVERSITY OF MICHIGAN) 24 November 1994 see the whole document --- -/--	1,11,15

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
3 October 1996	06. 11. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gac, G

International Application No
PCT/US 96/09526

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,93 14778 (VICAL INC.) 5 August 1993 see page 33; example 9 ---	17,19
A	METHODS CELL. BIOL., vol. 14, 1974, pages 33-71, XP002015103 POSTE G. ET AL.: "Lipid vesicles as carriers for introducing biological active materials into cells" see page 39 see page 42 - page 44 see page 52 -----	12-17

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/09526

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